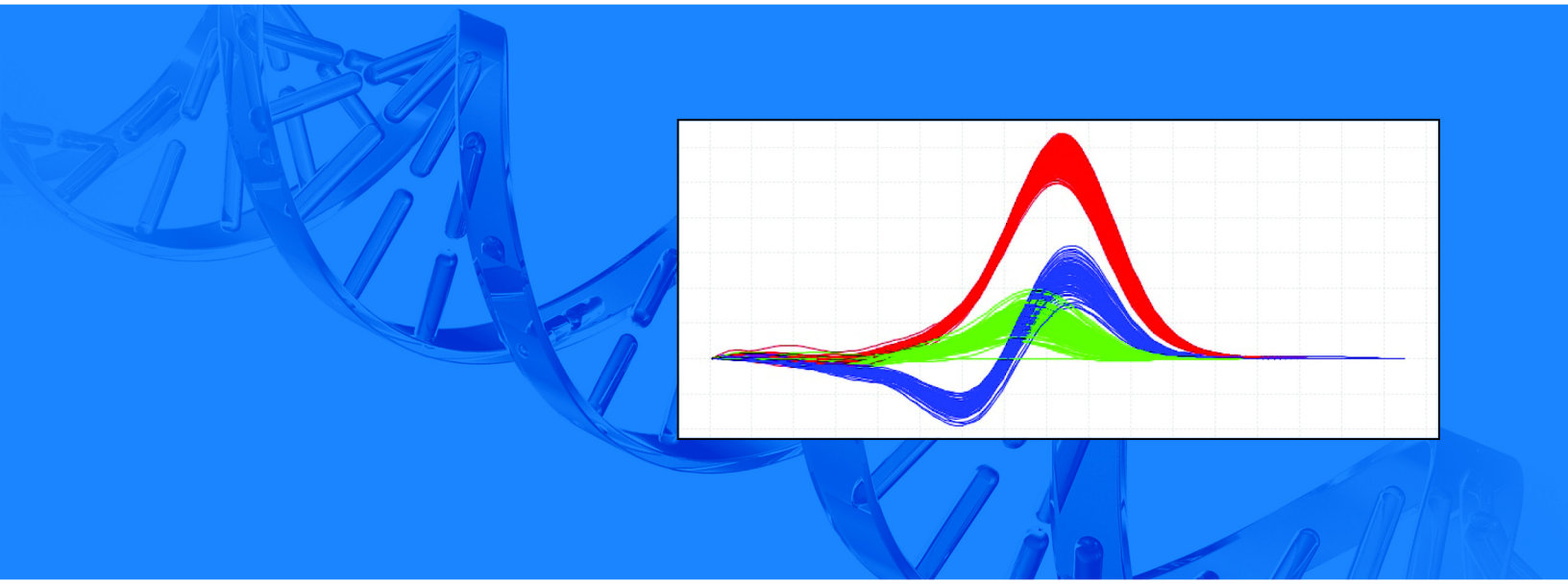
Applied Biosystems

Applied Biosystems High Resolution Melting



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TRADEMARKS:

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Contents

About This Guide

Purpose

This guide provides step-by-step procedures for:

- Calibrating an Applied Biosystems 7900HT Fast Real-Time PCR System or an Applied Biosystems 7500 Fast Real-Time PCR System to use the MeltDoctor™ HRM Dye
- Performing HRM experiments using MeltDoctor™ HRM Reagents: Designing the experiment, preparing the reactions, running the reactions, and reviewing the HRM data using HRM Software for HRM genotyping, HRM mutation scanning, and HRM methylation studies

This Getting Started Guide is designed to help you quickly learn to use MeltDoctor™ HRM Reagents and Applied Biosystems High Resolution Melting Software.

Prerequisites

This guide assumes that you have working knowledge of the:

- Microsoft® Windows® XP operating system
- Instrument system software for your Real-Time PCR System:
  - SDS Software v2.3 or later for the 7900HT Fast System
  - 7500 Software v2.0 or later for the 7500 Fast System
  - SDS Software v1.4 for the 7500 Fast System

Safety information

Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation or accurate chemistry kit use.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Except for IMPORTANTs, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol.

MSDSs

The MSDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining MSDSs, see "MSDSs" on page 139.

IMPORTANT! For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

1 Calibrate the Instrument

You must calibrate your instrument for the MeltDoctor™ HRM Dye before you can perform high resolution melting experiments using MeltDoctor™ HRM Reagents. Applied Biosystems recommends that you calibrate your instrument for each HRM dye that you are using or for each significant change in master mix composition.

This chapter describes how to calibrate an Applied Biosystems 7900HT Fast Real-Time PCR System or an Applied Biosystems 7500 Fast Real-Time PCR System for the MeltDoctor™ HRM Dye.

Note: If you use a different HRM dye, follow the calibration workflow in this chapter, but substitute the MeltDoctor™ HRM Dye with your HRM dye of choice. For component volumes to prepare the calibration plate, refer to the manufacturer's instructions.

This chapter contains instructions for calibrating the following Applied Biosystems Real-Time PCR Systems:

Real-Time PCR System	Page
7900H Fast system with SDS Software v2.3 or later	3
7500 Fast system with 7500 Software v2.0 or later	14
7500 Fast system with SDS Software v1.4	23

Section 1.1 Calibrate a 7900HT Fast system with SDS Software v2.3 or later

IMPORTANT! Perform the amplification run, custom dye calibration, and HRM calibration on the same day.

Perform a background calibration

Prepare the background calibration plate (page 4)

↓

Run the background calibration plate (page 4)

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Review the background calibration results (page 5)

↓

Amplify the DNA in the HRM calibration plate

Prepare the MeltDoctor™ HRM Calibration Plate (page 6)

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Run the HRM calibration plate to amplify the DNA (page 7)

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Verify that the HRM calibration samples amplified (page 9)

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Calibrate the instrument to use the MeltDoctor™ HRM Dye

Run the HRM calibration plate for the custom dye calibration (page 9)

↓

Review the pure dye spectra for irregularities (page 10)

↓

Perform a melt curve to generate the HRM calibration file

Run the HRM calibration plate for the HRM calibration (page 11)

↓

Verify that the dissociation curve contains only one Tm peak (page 13)

Perform a background calibration

IMPORTANT! Before you can run the HRM dye calibration plate on your instrument, you must perform a background calibration. The background calibration ensures that the background signal is even across the reaction plate and that variation is at a minimum.

Required materials for background calibration

- Appropriate reaction plate for your reaction block:
  - MicroAmp® Fast Optical 384-Well Reaction Plate with Barcode, 0.1 mL OR
  - MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL OR
  - MicroAmp® Optical 96-Well Reaction Plate with Barcode
- MicroAmp™ Optical Adhesive Film
- Deionized water
- Pipettors and pipette tips
- Centrifuge

Prepare the background calibration plate

1. Add deionized water into each well of an appropriate reaction plate for your reaction block:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

2. Seal the reaction plate with optical adhesive film, then spin the plate.

Run the background calibration plate

1. In the SDS Software, create a new run file for the background calibration:
   - Assay: **Background**
   - Container: Select the appropriate plate type:
     - **384 Wells Clear Plate** or
     - **96 Wells Clear Plate**
   - Template:
     - **Blank Template** for 384-well plates and standard 96-well plates
     - **Fast 96 Well Background Plate.sdt** for Fast 96-well plates
   - (Optional) Scan or enter the barcode

2. Set the sample volume in the **Instrument ▸ Thermal Cycler ▸ Thermal Profile** tab:
   - **20** µL for Fast 384-well plates and Fast 96-well plates
   - **50** µL for standard 96-well plates

3. Select the **Real-Time** tab, load the background calibration plate into the instrument, then start the run.

4. At the prompt, save the background calibration file:
   - Location: Create a folder called **HRMCalibrationFiles**.
   - File name: Use the convention:
     BackgroundCalibration_*<block type>*_*<today's date>*

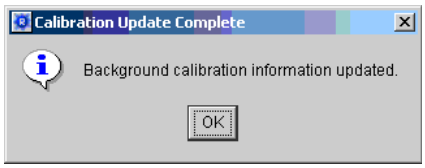
5. When the Run Complete dialog box opens, click **OK**.

6. Save the background calibration file, then unload the background calibration plate.

Review the background calibration results

1. Select **Analysis ▸ Extract Background**.

2. When the software displays the following message, click **OK**.

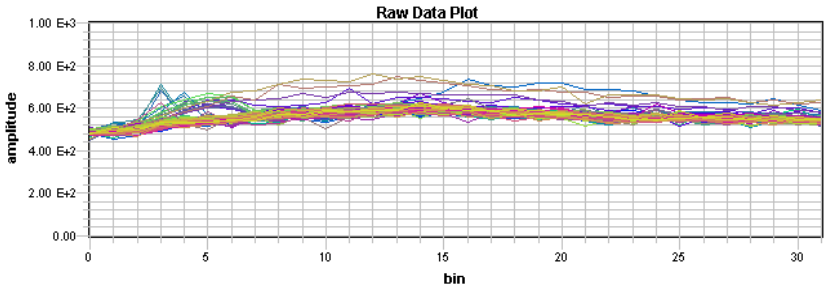


Note: If you receive a different message, refer to the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684).

3. In the toolbar, click  (**Hide/Show System Raw Data Pane**).

4. Select all wells in the plate grid, then view the Raw Data Plot and verify that there are no irregularities in the data (irregular spectral peaks).

IMPORTANT! If you find any irregularities in the data, refer to the procedures for decontaminating the sample block in the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684) The background calibration must be successful before you can perform a custom dye calibration.



5. Save and close the file.

6. Unload the background calibration plate.

Amplify the DNA in the HRM calibration plate

Required materials for HRM calibration

- Appropriate HRM calibration plate for your reaction block:

Reaction block	HRM calibration plate
384-well block	MeltDoctor™ HRM Calibration Plate, 384-Well
Fast 96-well block	MeltDoctor™ HRM Calibration Plate, Fast 96-Well
Standard 96-well block	Prepare your own HRM calibration plate.

Note: To prepare your own HRM calibration plate using the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Calibration Standard, follow the procedure in Appendix B on page 127.

- Centrifuge

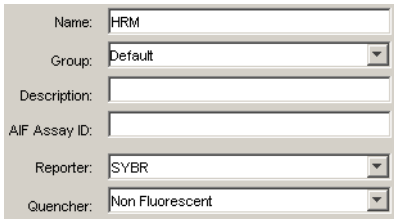
Prepare the MeltDoctor™ HRM Calibration Plate

1. Remove the MeltDoctor™ HRM Calibration Plate from the freezer, then allow it to thaw.

2. Spin the plate briefly.

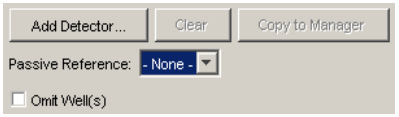
Run the HRM calibration plate to amplify the DNA

1. In the SDS Software, create a new run file for the amplification:
   - Assay: **Standard Curve (AQ)**
   - Container: Select the appropriate plate type:
     - **384 Wells Clear Plate** or
     - **96 Wells Clear Plate**
   - Template: **Blank Template**
   - (Optional) Scan or enter the barcode
2. Create and add the HRM calibration detector:
   a. In the Well Inspector, click **Add Detector**, then click **New**.
   b. Enter **HRM** for the Name, select **SYBR** for the Reporter, then click **OK**.



   c. In the Detector Manager, select the **HRM** detector, click **Copy to Plate Document**, then click **Done**.
3. Apply the HRM detector to the wells in the plate grid:
   a. Select all the wells in the plate grid.
   b. In the Well Inspector, select the **Use** checkbox for the HRM detector.

   c. With all the wells still selected, select **None** from the Passive Reference dropdown menu.



4. Set the thermal cycler protocol in the **Instrument ▸ Thermal Cycler ▸ Thermal Profile** tab:
   - Mode: **Standard**
   - Sample Volume (µL):
     - **20** µL for Fast 384-well plates and Fast 96-well plates
     - **50** µL for standard 96-well plates
   - Thermal profile:

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%

5. Select the **Real-Time** tab, load the HRM calibration plate into the instrument, then start the run.

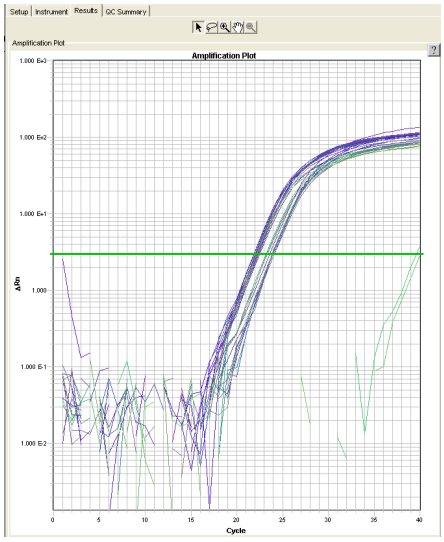
6. At the prompt, save the amplification file:
   - Location: HRMCalibrationFiles folder that you created when you performed the background calibration (see page 5).
   - File name: Use the convention:
     Amplification_<*block type*>_<*today's date*>

7. When the Run Complete dialog box opens, click **OK**.

8. Save the amplification file, then unload the HRM calibration plate.

Verify that the HRM calibration samples amplified

1. Click (**Analyze**).
2. View the Amplification Plot, then review the plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence
   - Standard deviation of C_T values ≤0.25.



Note: If the Amplification Plot looks abnormal, contact an Applied Biosystems representative to identify and resolve the problem.

3. Save and close the file.

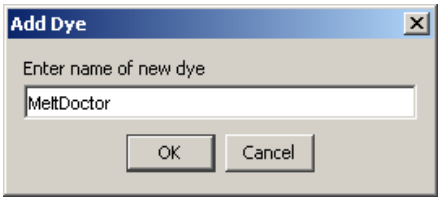
Calibrate the instrument to use the MeltDoctor™ HRM Dye

Perform a custom dye calibration for the MeltDoctor™ HRM Dye using the same MeltDoctor™ HRM Calibration Plate that you amplified on page 6.

Run the HRM calibration plate for the custom dye calibration

1. In the SDS Software, create a new run file for the custom dye calibration:
   - Assay: **Pure Spectra**
   - Container: Select the appropriate plate type:
     - **384 Wells Clear Plate** or
     - **96 Wells Clear Plate**
   - Template: **Blank Template**
   - (Optional) Scan or enter the barcode
2. Add the MeltDoctor™ HRM dye to the Dye Library:
   a. Select **Tools** ▸ **Dye Manager**, then click **New**.

b. Enter **MeltDoctor** for the name, then click **OK**.



c. Click **Done** to exit the Dye Manager.

3. Apply the MeltDoctor dye to the plate grid:

   a. Select all the wells in the plate grid.

   b. From the Dyes dropdown list, select **MeltDoctor**.

4. Select the **Instrument** ▸ **Thermal Cycler** ▸ **Thermal Profile** tabs, then set the sample volume:
   - **20** µL for Fast 384-well plates and Fast 96-well plates
   - **50** µL for standard 96-well plates

5. Select the **Real-Time** tab, spin the HRM calibration plate briefly, load the plate into the instrument, then start the run.

6. At the prompt, save the custom dye calibration file:
   - Location: HRMCalibrationFiles folder that you created when you performed the background calibration (see page 5).
   - File name: Use the convention:
     CustomDye_*<block type>*_*<today's date>*

7. When the Run Complete dialog box opens, click **OK**.

8. Save the custom dye calibration file, then unload the HRM calibration plate.

Review the pure dye spectra for irregularities

1. Select **Analysis** ▸ **Extract Pure Dye Wizard**.

2. Follow the instructions in the Pure Dye Wizard to extract the pure dye spectra. In each screen, inspect the spectra for shifts in peak location. When complete, the software displays a message reporting the extraction of the pure dyes.

Note: If you find any irregularities in the data, refer to the *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684). You cannot complete the HRM calibration until the custom dye calibration passes.

3. Save the custom dye calibration file, unload the HRM calibration plate, then spin the plate briefly.

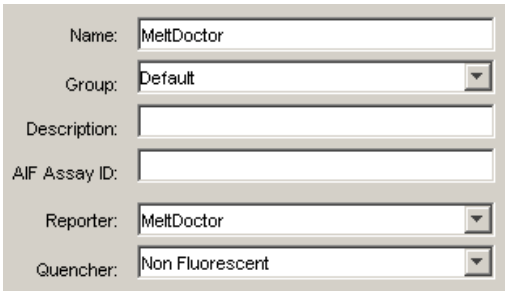
Perform a melt curve to generate the HRM calibration file

Use the HRM calibration plate a third time to perform a melt curve and generate an HRM calibration file for the Applied Biosystems High Resolution Melting Software (HRM Software).

Run the HRM calibration plate for the HRM calibration

1. In the SDS Software, create a new run file for the HRM calibration:
   - Assay: **Standard Curve (AQ)**
   - Container: Select the appropriate plate type:
     - **384 Wells Clear Plate** or
     - **96 Wells Clear Plate**
   - Template: **Blank Template**
   - (Optional) Scan or enter the barcode

2. Create and add the MeltDoctor detector to the plate document:
   a. In the Well Inspector, click **Add Detector**, then click **New**.
   b. Enter **MeltDoctor** for the Name, select **MeltDoctor** for the Reporter, then click **OK**.



   c. In the Detector Manager, select the MeltDoctor detector, click **Copy to Plate Document**, then click **Done**.

3. Apply the MeltDoctor detector to the wells in the plate grid:
   a. Select all the wells in the plate grid.
   b. In the Well Inspector, select the **Use** checkbox for the MeltDoctor detector.

c. With all the wells still selected, select **None** from the Passive Reference dropdown menu.

4. Select the **Instrument** ▸ **Thermal Cycler** ▸ **Thermal Profile** tabs, then set the thermal cycler protocol:
   - Mode: **Standard**
   - Sample Volume (µL):
     - **20** µL for Fast 384-well plates and Fast 96-well plates
     - **50** µL for standard 96-well plates
   - Thermal profile:

Stage	Step	Temp	Time	Ramp rate
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%

5. Select the **Real-Time** tab, spin the HRM calibration plate briefly, load the plate into the instrument, then start the run.

6. At the prompt, save the melt curve file:
   - Location: HRMCalibrationFiles folder that you created when you performed the background calibration (see page 5).
   - File name: Use the convention:
     HRMCalibration_MeltDoctorDye_<*instrument info*>_<*today's date*>

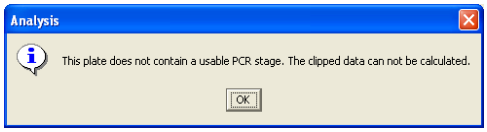
   IMPORTANT! Make sure you include instrument information (instrument model number, plate type, and software version) in the file name so you can verify that the HRM calibration file and the HRM experiment file are run on the same instrument. If you have multiple instruments, include information to identify the instrument (for example, an instrument name).

7. When the Run Complete dialog box opens, click **OK**.

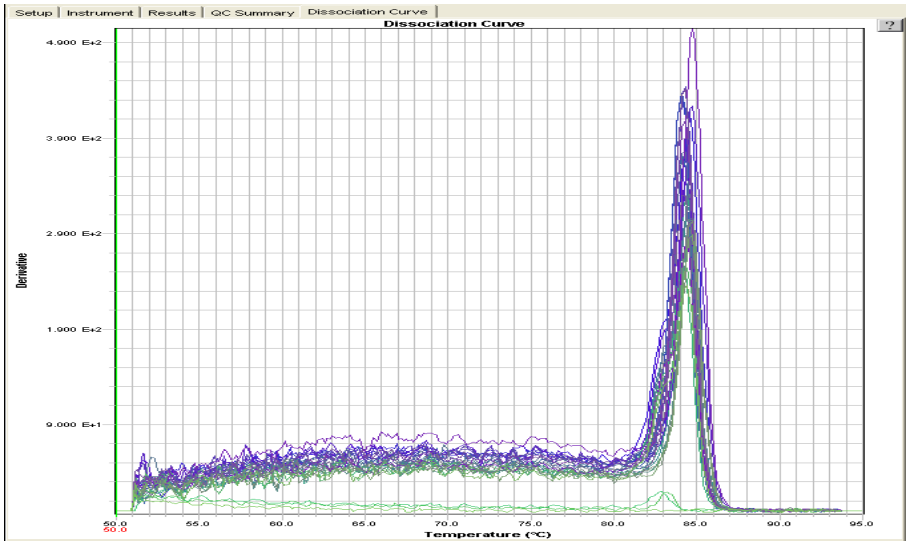
8. Save the HRM calibration file, then unload the HRM calibration plate.

Verify that the dissociation curve contains only one Tm peak

1. Click (**Analyze**).
2. If you receive the following message, click **OK**.



3. View the Dissociation Curve, then verify that the curve contains only 1 Tm peak, as in the example below.



Note: If the Dissociation Curve contains more than 1 Tm Peak, more than one PCR product was produced. Contact an Applied Biosystems representative to identify and resolve the problem.

4. Save and close the file, then unload the HRM dye calibration plate.

IMPORTANT! When you start the HRM software for the first time, you will be prompted to select the default HRM calibration file. Select this file (**HRMCalibration_MeltDoctorDye_*<instrumentinfo>*_*<today's date>***).

Section 1.2 Calibrate a 7500 Fast system with 7500 Software v2.0 or later

IMPORTANT! Perform the amplification run, custom dye calibration, and HRM calibration on the same day.

Perform a background calibration

Prepare the background calibration plate (page 15)

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Run the background calibration plate (page 15)

↓

Review the background calibration results (page 16)

↓

Amplify the DNA in the HRM calibration plate

Prepare the MeltDoctor™ HRM Calibration Plate (page 17)

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Run the HRM calibration plate to amplify the DNA (page 17)

↓

Verify that the HRM calibration samples amplified (page 18)

↓

Calibrate the instrument to use the MeltDoctor™ HRM Dye

Run the HRM calibration plate for the custom dye calibration (page 19)

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Review the custom dye calibration results (page 19)

↓

Perform a melt curve to generate the HRM calibration file

Run the HRM calibration plate for the HRM calibration (page 20)

↓

Verify that the Melt Curve contains only one Tm peak (page 21)

Perform a background calibration

IMPORTANT! Before you can run the HRM dye calibration plate on your instrument, you must perform a background calibration. The background calibration ensures that the background signal is even across the reaction plate and that variation is at a minimum.

Required materials for background calibration

- MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL
- MicroAmp™ Optical Adhesive Film
- Deionized water
- Pipettors and pipette tips
- Centrifuge

Prepare the background calibration plate

1. Add 20 µL deionized water into each well of a Fast 96-well reaction plate.
2. Seal the reaction plate with optical adhesive film, then spin the plate.

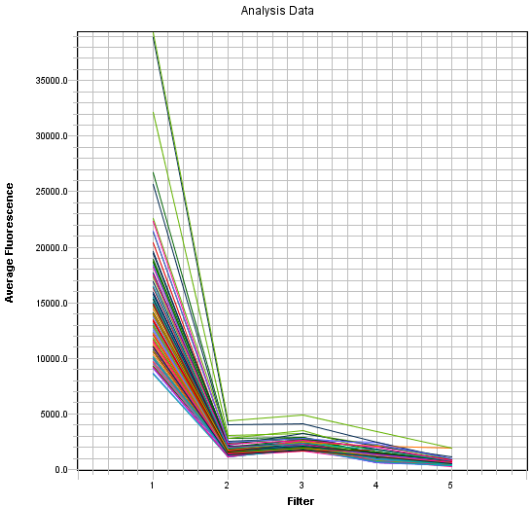
Run the background calibration plate

1. In the 7500 Software, select **Instrument** ▸ **Instrument Maintenance Manager**, then in the navigation pane, select **Background**.
2. Click **Start Calibration**, then follow the instructions in the Setup screen, but use the background calibration plate you prepared above.
3. In the Run screen, click **START RUN**.
   When the run is complete, the Analysis screen is automatically displayed.

Review the background calibration results

1. In the Analysis screen, verify that the background calibration passed.

   Note: If the background calibration failed, refer to the procedures for cleaning the sample block in the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide* (PN 4387777). The background calibration must pass before you can perform a custom dye calibration.



2. Finish the background calibration and close the Instrument Maintenance Manager. The software saves the background calibration file.

3. Unload the background calibration plate.

Amplify the DNA in the HRM calibration plate

Required materials for HRM calibration

- MeltDoctor™ HRM Calibration Plate, Fast 96-Well

  Note: To prepare your own HRM calibration plate using the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Calibration Standard, follow the procedure in Appendix B on page 127.

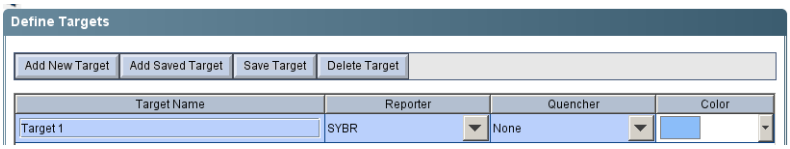
- Centrifuge

Prepare the MeltDoctor™ HRM Calibration Plate

1. Remove the Fast 96-Well MeltDoctor™ HRM Calibration Plate from the freezer, then allow it to thaw.
2. Spin the plate briefly.

Run the HRM calibration plate to amplify the DNA

1. In the 7500 Software, create a new experiment for the amplification:
   - Experiment Name – Use the convention: **Amplification_*<today's date>***
   - Instrument – **7500 Fast (96 Wells)**
   - Experiment type – **Quantitation - Standard Curve**
   - Reagents – **SYBR® Green Reagents**
   - Ramp speed – **Standard (~ 2 hours to complete a run)**
2. In the Plate Setup ▸ Define Targets and Samples tab, define the calibration target as **Target 1** for the target name and **SYBR** for the reporter.



3. In the Plate Setup ▸ Assign Targets and Samples tab, assign the Target 1 target to the wells in the plate grid:
   a. Select all the wells in the plate grid.
   b. Select the **Assign** checkbox for Target 1, then select U (**Unknown**) as the Task.

   c. Select **None** as the passive reference.

4. In the **Setup ▸ Run Method** tab, set the thermal cycler conditions:
   - Reaction Volume Per Well: **20** µL
   - Thermal profile:

Stage	Step	Temp	Time
Holding	Enzyme activation	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	15 sec
	Anneal/extend	60 °C	1 min

5. Load the HRM calibration plate into the instrument, then start the run.

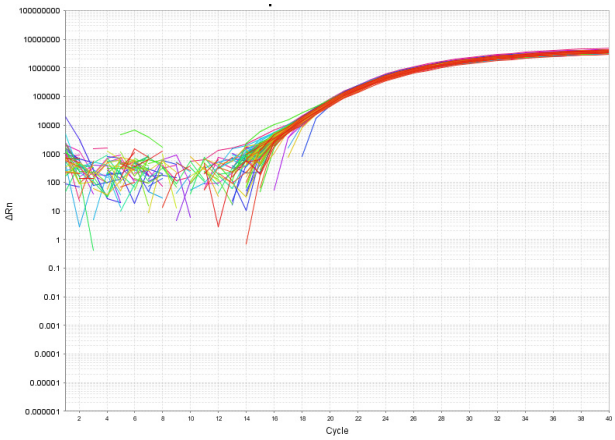
6. At the prompt, save the amplification file:
   - Location: Create a folder called **HRMCalibrationFiles**.
   - File name: Use the convention **Amplification_*<today's date>***

   When the run is complete, the Analysis screen is automatically displayed.

7. Unload the HRM calibration plate.

Verify that the HRM calibration samples amplified

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence
   - Standard deviation of C_T values ≤0.25.



Note: If the Amplification Plot looks abnormal, contact an Applied Biosystems representative to identify and resolve the problem.

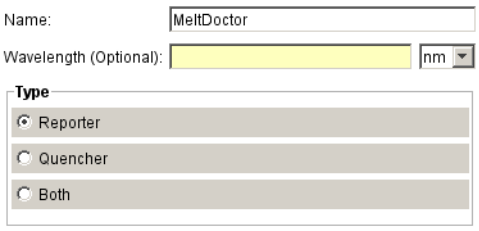
2. Save and close the file.

Calibrate the instrument to use the MeltDoctor™ HRM Dye

Perform a custom dye calibration for the MeltDoctor™ HRM Dye using the same MeltDoctor™ HRM Calibration Plate that you amplified on page 18.

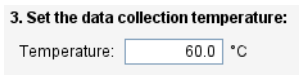
Run the HRM calibration plate for the custom dye calibration

1. In the 7500 Software, select **Instrument ▸ Instrument Maintenance Manager**, then in the navigation pane, select **Custom Dye Calibration**.
2. Click **Start Calibration**, then follow the instructions in the Setup screen:
   a. In step 1, for the custom dye calibration plate, use the amplified MeltDoctor™ HRM Calibration Plate.
   b. Add the HRM dye to the Dye Library: Click **New Dye**, then enter **MeltDoctor** for the name, select **Reporter** as the dye type, and click **OK**.



   c. In step 2, select **MeltDoctor** as the Dye Name.

   d. In step 3, set the temperature to **60 °C (default)**.



   e. Spin the HRM calibration plate briefly, load the plate into the instrument, select the checkbox **The custom dye plate is loaded into the instrument**, then click **Next**.
3. In the Run screen, click **START RUN**.
4. When the run is complete, unload the HRM calibration plate, then click **Next** to view the Analysis screen.

Review the custom dye calibration results

1. In the Analysis screen, verify that the custom dye calibration passed.

   Note: If the custom dye calibration failed, refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide* (PN 4387777). You cannot complete the HRM calibration until the custom dye calibration passes.

2. Finish the custom dye calibration and close the Instrument Maintenance Manager. The software saves the custom dye calibration file.

Perform a melt curve to generate the HRM calibration file

Use the HRM calibration plate a third time to perform a melt curve and generate an HRM calibration file for the Applied Biosystems High Resolution Melting Software (HRM Software).

Run the HRM calibration plate for the HRM calibration

1. In the 7500 Software, create a new experiment for the HRM calibration:
   - Experiment Name – Use the convention **HRMCalibration_MeltDoctorDye_*<instrument info>*_*<today's date>***
   - Instrument – **7500 Fast (96 Wells)**
   - Experiment type – **Melt Curve**
   - Reagents – **Other**
   - Ramp speed – **Fast**

2. In the Plate Setup ▸ Define Targets and Samples tab, define the calibration target as **Target 1** for the target name and **MeltDoctor** for the reporter.

3. In the Plate Setup ▸ Assign Targets and Samples tab, assign Target 1 to the wells in the plate grid:
   a. Select all the wells in the plate grid.
   b. Select the **Assign** checkbox for Target 1, then select U (**Unknown**) as the Task.

   c. Select **None** as the passive reference.

4. Set the thermal cycler protocol in the **Instrument ▸ Thermal Profile** tab:
   - Reaction Volume Per Well – **20** µL
   - Thermal profile:

Stage	Step	Temp	Time
Melt curve/dissociation	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melting	95 °C	15 sec
	Anneal	60 °C	15 sec

   - Click **Select/View Filters**, then select only **Filter-1**

5. Spin the HRM calibration plate briefly, load the plate into the instrument, then start the run.

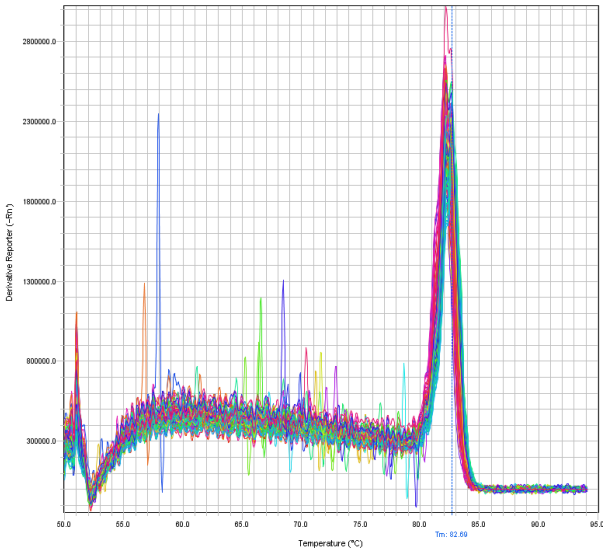
6. At the prompt, save the HRM calibration file:
   - Location: HRMCalibrationFiles folder that you created when you amplified the HRM calibration plate (see page 18).
   - File name: Use the convention:
     HRMCalibration_MeltDoctorDye_<*instrument info*>_<*today's date*>

   IMPORTANT! Make sure you include instrument information (instrument model number, plate type, and software version) in the file name so you can verify that the HRM calibration file and the HRM experiment file are run on the same instrument. If you have multiple instruments, include information to identify the instrument (for example, an instrument name).

   When the run is complete, the Analysis screen is automatically displayed.

Verify that the Melt Curve contains only one Tm peak

1. Verify that the Melt Curve contains only 1 Tm peak, as in the example below.



   Note: If the Melt Curve contains more than 1 Tm Peak, more than one PCR product was produced. Contact an Applied Biosystems representative to identify and resolve the problem.

2. Save and close the file, then unload the HRM dye calibration plate.

   IMPORTANT! When you start the HRM software for the first time, you will be prompted to select the default HRM calibration file. Select this file (**HRMCalibration_MeltDoctorDye_<*instrumentinfo*>_<*today's date*>**).

Section 1.3 Calibrate a 7500 Fast system with SDS Software v1.4

IMPORTANT! Perform the amplification run, custom dye calibration, and HRM calibration on the same day.

Perform a background calibration

Prepare the background calibration plate (page 24)

↓

Run the background calibration plate (page 24)

↓

Review the background calibration results (page 24)

↓

Amplify the DNA in the HRM calibration plate

Prepare the MeltDoctor™ HRM Calibration Plate (page 26)

↓

Run the HRM calibration plate to amplify the DNA (page 26)

↓

Verify that the HRM calibration samples amplified (page 28)

↓

Calibrate the instrument to use the MeltDoctor™ HRM Dye

Run the HRM calibration plate for the custom dye calibration (page 29)

↓

Review the pure dye spectra for irregularities (page 29)

↓

Perform a melt curve to generate the HRM calibration file

Run the HRM calibration plate for the HRM calibration (page 31)

↓

Verify that the Dissociation Curve contains only one Tm peak (page 33)

Perform a background calibration

IMPORTANT! Before you can run the HRM dye calibration plate on your instrument, you must perform a background calibration. The background calibration ensures that the background signal is even across the reaction plate and that variation is at a minimum.

Required materials for background calibration

- MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL
- MicroAmp™ Optical Adhesive Film
- Deionized water
- Pipettors and pipette tips
- Centrifuge

Prepare the background calibration plate

1. Add 20 µL deionized water into each well of a Fast 96-well reaction plate.
2. Seal the reaction plate with optical adhesive film, then spin the plate.

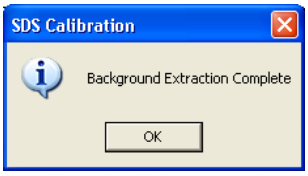
Run the background calibration plate

1. In the SDS Software, create a new run file for the background calibration:
   - Assay: **Background**
   - Container: **96-Well Clear**
   - Template: **Blank Document**
2. Select the **Instrument** tab, load the calibration plate into the instrument, then start the run.
3. At the prompt, save the background calibration file:
   - Location: Create a folder called **HRMCalibrationFiles**.
   - File name: Use the convention: **BackgroundCalibration_*<today's date>***
4. When the *Run completed successfully* message appears, click **OK**.
5. Save the background calibration file, then unload the background calibration plate.

Review the background calibration results

1. Review the background calibration results:
   a. Click  (or select **Analysis** ▸ **Extract Background**).

b. When the software displays the following message, click **OK**.

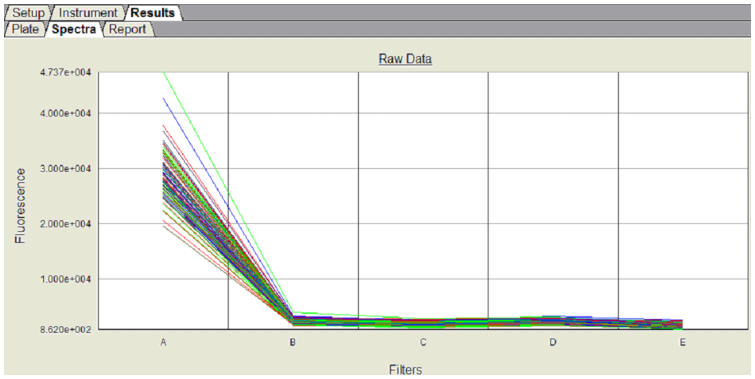


Note: If you receive a different message, refer to the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide*.

c. Select the **Results** ▸ **Spectra** tabs, then select all wells in the plate grid.

d. Inspect the raw data to verify that there are no irregularities in the data (irregular spectral peaks).

Note: If you find any irregularities in the data, refer to the procedures for cleaning the sample block in the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide* (PN 4347828). The background calibration must be successful before you can perform a custom dye calibration.



2. Save and close the file.

3. Unload the background calibration plate.

Amplify the DNA in the HRM calibration plate

Required materials for HRM calibration

- MeltDoctor™ HRM Calibration Plate, Fast 96-Well

Note: To prepare your own HRM calibration plate using the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Calibration Standard, follow the procedure in Appendix B on page 127.

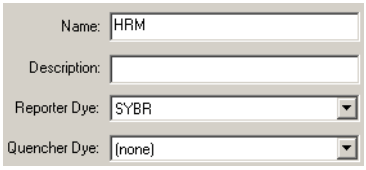
- Centrifuge

Prepare the MeltDoctor™ HRM Calibration Plate

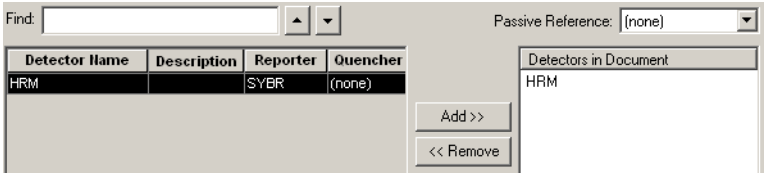
1. Remove the Fast 96-Well MeltDoctor™ HRM Calibration Plate from the freezer, then allow it to thaw.
2. Spin the plate briefly.

Run the HRM calibration plate to amplify the DNA

1. In the SDS Software, create a new run file for the amplification:
   - Assay: **Standard Curve (Absolute Quantitation)**
   - Container: **96-Well Clear**
   - Template: **Blank Document**
2. Create and add a detector to the plate document:
   a. Click **New Detector**.
   b. Enter **HRM** for the Name, select **SYBR** for the Reporter Dye, then click **OK**.

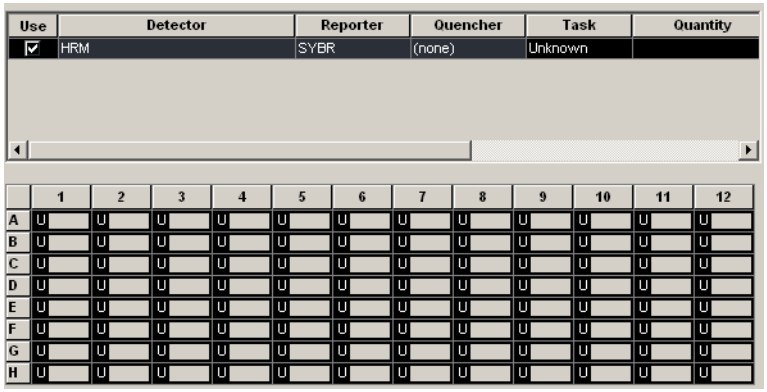


   c. Select **HRM** from the table, then click **Add**.
   d. From the Passive Reference dropdown menu, select **(none)**, then click **Next**.



3. Apply the detector to the wells in the plate grid:
   a. Select all the wells in the plate grid.

b. Select the **Use** checkbox for the HRM detector.



4. Set the thermal cycler protocol in the **Instrument ▸ Thermal Profile** tab:
   - Thermal profile:

Stage	Step	Temp	Time
Holding	Enzyme activation	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	15 sec
	Anneal/extend	60 °C	1 min

   - Sample Volume (µL): **20**
   - Run Mode: **Fast 7500**

5. Load the HRM calibration plate into the instrument, then start the run.

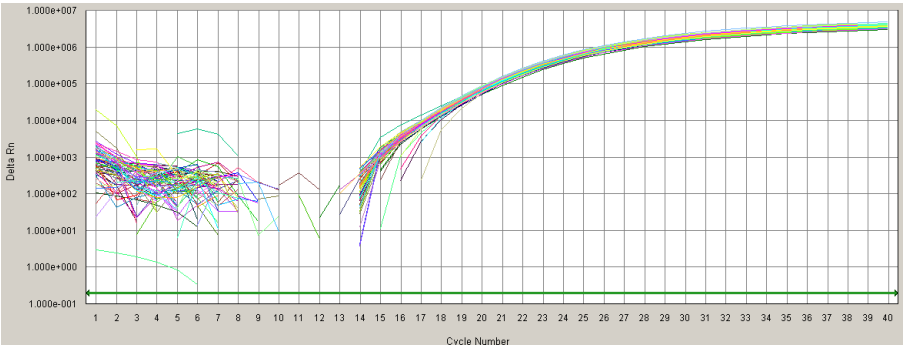
6. At the prompt, save the amplification file:
   - Location: HRMCalibrationFiles folder that you created when you performed the background calibration (see page 24).
   - File name: Use the convention:
     Amplification_*<today's date>*

7. When the *Run completed successfully* message appears, click **OK**.

8. Save the amplification file, then unload the HRM calibration plate.

Verify that the HRM calibration samples amplified

1. Click (**Analyze**), then select the **Results** tab.
2. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence
   - Standard deviation of C_T values ≤0.25.



Note: If the Amplification Plot looks abnormal, contact an Applied Biosystems representative to identify and resolve the problem.

3. Save and close the file.

Calibrate the instrument to use the MeltDoctor™ HRM Dye

Perform a custom dye calibration for the MeltDoctor™ HRM Dye using the same MeltDoctor™ HRM Calibration Plate that you amplified on page 17.

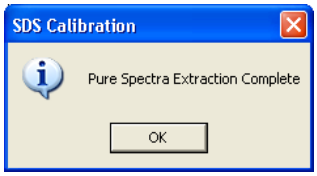
Run the HRM calibration plate for the custom dye calibration

1. In the SDS Software, create a new run file for the custom dye calibration:
   - Assay: **Pure Spectra**
   - Container: **96-Well Clear**
   - Template: **Blank Document**
2. In the Pure Spectra Calibration Manager, set up the custom dye calibration:
   a. Click **Add Dye**.
   b. Enter **MeltDoctor** for the name, then click **OK**.
   c. Select **MeltDoctor** from the dye list, then click **Calibrate**.

   Note: If you are prompted to disconnect the plate document, click **Yes**.
3. Apply the MeltDoctor dye to the plate grid:
   a. Select all the wells in the plate grid.
   b. From the Dyes dropdown list, select **MeltDoctor**.
4. Spin the HRM calibration plate briefly, load the plate into the instrument, then click **Yes** to start the custom dye calibration.
5. When the run is complete, click **Finish**. The software automatically saves the HRM dye data to a calibration file on the computer hard drive.
6. Unload the HRM calibration plate.

Review the pure dye spectra for irregularities

1. Click  (or select **Analysis** ▸ Extract Pure Spectra).
2. When the software displays the following message, click **OK**.

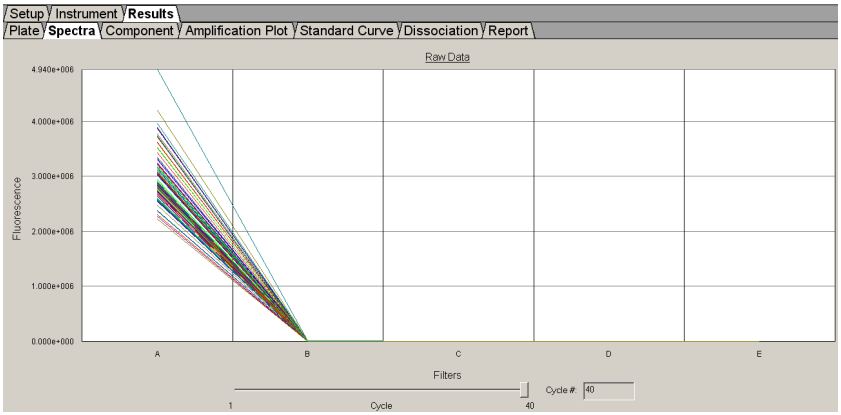


Note: If you receive a different message, refer to *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide*.

3. Select the **Results** ▸ **Spectra** tabs, then select all wells in the plate grid.

4. Verify that the peak of the MeltDoctor™ Dye appears in Filter A.

Note: If you find any irregularities in the data, refer to the Applied *Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide* (PN 4347828). You cannot complete the HRM calibration until the custom dye calibration passes.



5. Close the custom dye calibration file, then unload the HRM calibration plate.

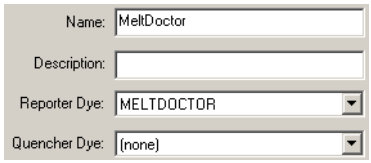
Perform a melt curve to generate the HRM calibration file

Use the HRM calibration plate a third time to perform a melt curve and generate an HRM calibration file for the Applied Biosystems High Resolution Melting Software (HRM Software).

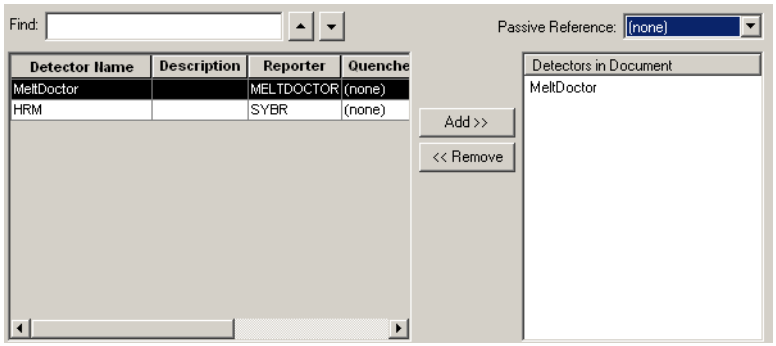
Run the HRM calibration plate for the HRM calibration

1. In the SDS Software, create a new run file for the HRM calibration:
   - Assay: **Standard Curve (Absolute Quantitation)**
   - Container: **96-Well Clear**
   - Template: **Blank Document**

2. Create and add an HRM detector to the plate document:
   a. Click **New Detector**.
   b. Enter **MeltDoctor** for the Name, select **MELTDOCTOR** for the Reporter Dye, then click **OK**.

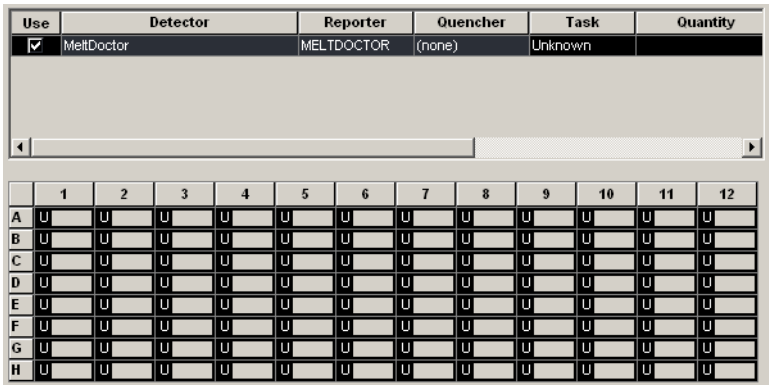


   c. Select **MeltDoctor** from the table, then click **Add**.
   d. From the Passive Reference dropdown menu, select **(none)**, then click **Next**.



3. Apply the detector to the wells in the plate grid:
   a. Select all the wells in the plate grid.

b. Select the **Use** checkbox for the MeltDoctor detector.



4. Set the thermal cycler protocol in the **Instrument ▸ Thermal Profile** tab:
   - Thermal profile:

Stage	Step	Temp	Time
Melt curve/dissociation	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melting	95 °C	15 sec
	Anneal	60 °C	15 sec

   - Sample Volume (µL): **20**
   - Run Mode: **Fast 7500**
   - Expert Mode: Select the checkbox, then select **Filter A**

5. Spin the HRM calibration plate briefly, load the plate into the instrument, then start the run.

6. At the prompt, save the HRM calibration file:
   - Location: HRMCalibrationFiles folder that you created when you performed the background calibration (see page 24).
   - File name: Use the convention:
     HRMCalibration_MeltDoctorDye_*<instrument info>_<today's date>*

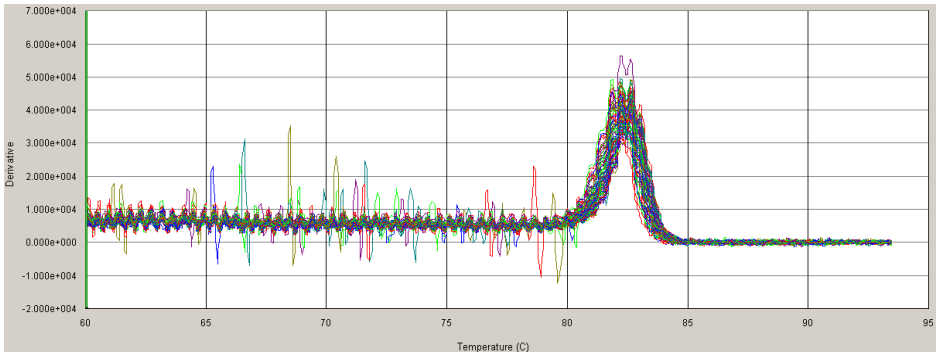
     IMPORTANT! Make sure you include instrument information (instrument model number, plate type, and software version) in the file name so you can verify that the HRM calibration file and the HRM experiment file are run on the same instrument. If you have multiple instruments, include information to identify the instrument (for example, an instrument name).

7. When the *Run completed successfully* message appears, click **OK**.

8. Save the HRM calibration file, then unload the HRM calibration plate.

Verify that the Dissociation Curve contains only one Tm peak

1. Click (**Analyze**), then select the **Results** tab.
2. Verify that the Dissociation Curve contains only 1 Tm peak, as in the example below.



Note: If the Dissociation Curve contains more than 1 Tm Peak, more than one PCR product was produced. Contact an Applied Biosystems representative to identify and resolve the problem.

3. Save and close the file, then unload the HRM dye calibration plate.

IMPORTANT! When you start the HRM software for the first time, you will be prompted to select the default HRM calibration file. Select this file (**HRMCalibration_MeltDoctorDye_<*instrumentinfo*>_<*today's date*>**).

2 Perform an HRM Experiment

Use MeltDoctor™ HRM Reagents and Applied Biosystems High Resolution Melting Software (HRM Software) to generate and analyze high-resolution melting curves from HRM reactions run on a 7500 Fast Real-Time PCR System or a 7900HT Fast Real-Time PCR System.

This chapter provides general instructions for performing an HRM experiment, using the MeltDoctor™ HRM Positive Control Kit as an example.

Perform an HRM Experiment

Design the HRM experiment (below)

↓

Prepare the HRM reactions (page 36)

↓

Amplify and melt the DNA (page 40)

↓

Review the high-resolution melting data (page 56)

Design the HRM experiment

Design and order the primers

Using Primer Express® Software v3.0 or later, design the primers to amplify the sequence of interest. Order the primers from the Applied Biosystems Store.

If you are using the MeltDoctor™ HRM Positive Control Kit, the kit contains primers designed to amplify the alleles in the positive control DNA. You do not need to design primers to use the Positive Control Kit.

1. Design the primers so that they meet these guidelines:

Design attribute	Design guidelines
Amplicon	Length is less than 250 basepairs
Primer length	~20 bases each
Tm	58 °C to 60 °C (Optimal Tm is 59 °C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

2. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Ordering custom primers" on page 128.

Select controls

Include controls for each target sequence in your HRM experiment:

- At least one negative control
- At least one positive control to represent each expected variant (for genotyping experiments)

  Run 3 to 5 replicates for each expected variant to improve your results. Running multiple positive controls allows you to more effectively define the natural spread or variation within different samples of the same sequence, or within replicates of the same genotype.
- At least one wild type control (for mutation scanning experiments)

  Run up to 5 replicates for each wild type control to improve your results. Running multiple wild type controls allows you to more effectively define the natural spread or variation within the normal population.

Prepare the HRM reactions

Combine purified genomic DNA templates with MeltDoctor™ HRM Master Mix and primers to amplify the target sequence.

With the MeltDoctor™ HRM Positive Control Kit, combine positive control Allele DNA with the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Primer Mix to amplify the alleles.

Note: If you are using the MeltDoctor™ HRM Reagent Kit instead of the MeltDoctor™ HRM Master Mix, see page 129 for reaction component volumes.

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your Real-Time PCR instrument
- MicroAmp™ Optical Adhesive Film
- For the example experiment using the MeltDoctor™ HRM Positive Control Kit, components from the kit:
  - MeltDoctor™ HRM Primer Mix (20×)
  - MeltDoctor™ HRM Allele A DNA (20×)
  - MeltDoctor™ HRM Allele G DNA (20×)
  - MeltDoctor™ HRM Allele A/G DNA (20×)
- For your own HRM experiments:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- MeltDoctor™ HRM Master Mix
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Prepare the HRM reactions

Prepare the reactions for each replicate group separately, then transfer the reactions to a reaction plate appropriate for your instrument.

To prepare HRM reactions for your own HRM experiments:

HRM experiment type	See page
HRM genotyping experiments	page 68
HRM mutation scanning experiments	page 80
HRM methylation studies	page 97

For information about using the MeltDoctor™ HRM Reagent Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 129.

1. Prepare the negative control reactions in an appropriately sized, labeled tube:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10 µL	33.0 µL	25.0 µL	82.5 µL
MeltDoctor™ HRM Primer Mix (20×)	1 µL	3.3 µL	2.5 µL	8.25 µL
Deionized water	9 µL	29.7 µL	22.5 µL	74.25 µL
Total volume	**20 µL**	**66 µL**	**50 µL**	**165 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

2. Prepare the reactions using the Positive Control Kit in separate appropriately sized, labeled tubes:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10 µL	33.0 µL	25.0 µL	82.5 µL
One type of allele DNA: • MeltDoctor™ HRM Allele A DNA (20×) • MeltDoctor™ HRM Allele G DNA (20×) • MeltDoctor™ HRM Allele A/G DNA (20×)	1 µL	3.3 µL	2.5 µL	8.25 µL
MeltDoctor™ HRM Primer Mix (20×)	1 µL	3.3 µL	2.5 µL	8.25 µL
Deionized water	8 µL	26.4 µL	20.0 µL	66.0 µL
Total volume	**20 µL**	**66 µL**	**50 µL**	**165 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

3. Vortex the reactions to mix, then spin the tubes briefly.

4. Prepare a reaction plate appropriate for your instrument:

   a. Pipet each reaction replicate to the appropriate wells of the optical reaction plate:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.

   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4 °C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data:

Run a 384-well plate on a 7900HT Fast instrument

Create and set up a new run file for the HRM run

1. In the SDS Software, create a new run file for the HRM run:
   - Assay: **Standard Curve (AQ)**
   - Container: **384 Wells Clear Plate**
   - Template: **Blank Template**
   - (Optional) Scan or enter the barcode
2. For each target sequence in the reaction plate, create and add a detector to the plate document:
   a. Click **Add Detector**, then look for the detector that represents the target sequence in your HRM reactions and the HRM dye that you are using to detect the variants.
   b. If the appropriate detector is not listed, click **New**, then complete the Add Detector dialog box:
      - Name: Enter a name for the detector to identify the target sequence
      - Reporter: **MeltDoctor**
      - Quencher: **Non Fluorescent**

      Note: SDS Software uses the term *detector*; the HRM software uses the term *target*. When completing the Add Detector dialog box, note that the name you assign to the detector will appear as the target name in the HRM software.

   c. Select the appropriate detector, click **Copy To Plate Document**, then click **Done**.
   d. If not already selected, select the **Setup** tab to view the Well Inspector.

e. In the plate grid, select the wells that contain the detector, including negative controls, positive controls, and sample reactions, then select the **Use** checkbox for the detector that is in those wells.

Note: If you are running multiple assays, make sure you create and add a detector for each target sequence in the plate. The HRM Software will automatically separate the wells into different assays according to the detector assigned to the well.

3. Set up the file to match the layout of your reaction plate:
   - Indicate which wells in the reaction plate contain negative controls. Select the negative control wells in the plate grid, then select **NTC** from the Task dropdown menu.

     Note: Negative controls are needed to control for the amplification, but they are not appropriate for HRM analysis. Make sure you select the NTC task for negative controls so that the HRM Software will automatically omit the negative controls from the HRM analysis.

   - Add sample names. For each sample, select wells in the plate grid then enter a name for the sample.

4. Set the Passive Reference to None: Select the wells in the plate grid, then select **None** from the Passive Reference dropdown menu.

Run the reaction plate to amplify and melt the DNA

1. Set the thermal cycler protocol on the Thermal Profile and Ramp Rate tabs:
   - Mode: **Standard**
   - Sample Volume (µL): **20**
   - Thermal profile:

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%

2. Select the **Real-Time** tab, load the reaction plate into the instrument, then start the run.

3. At the prompt, browse to the location where to save the file, enter a name for your experiment, then click **Save**.

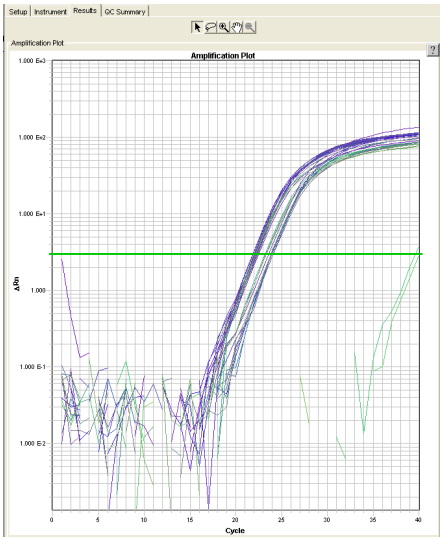
4. When the Run Complete dialog box opens, click **OK**.

5. Save the file, then unload the reaction plate.

Verify that the samples amplified and review the Tm peaks

1. Click (**Analyze**), then select the **Results** tab.

2. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: If the Amplification Plot looks abnormal, refer to Chapter 6, "Troubleshooting HRM Experiments" on page 115 to identify and resolve the problem.

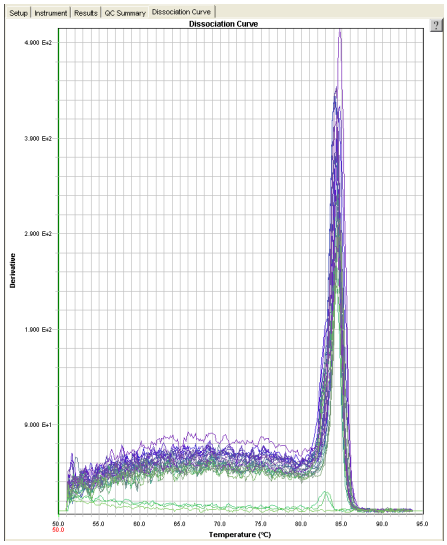
3. Look for outliers with C_T values that differ from replicates by more than 2.

Note: Note which wells are outliers. The outliers may produce erroneous HRM results.

4. Verify that the Dissociation Curve shows no unexpected Tm peaks.

   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 Tm peak because of the large differences between the different sequence types.

   Note: Unexpected peaks at low temperatures may indicate contamination, primer dimers, or non-specific amplification.



5. Save and close the file.

6. Proceed with "Review the high-resolution melting data" on page 56.

Run a 96-well plate on a 7900HT Fast instrument

Note: If you are performing your experiment on the 7900HT instrument using a 96-well reaction plate, perform the amplification and melt curve in separate runs so you can spin the plate before you perform the melt curve.

Create and set up a new run file for the amplification

1. In the SDS Software, create a new run file for the amplification:
   - Assay: **Standard Curve (AQ)**
   - Container: **96 Wells Clear Plate**
   - Template: **Blank Template**
   - (Optional) Scan or enter the barcode

2. For each target sequence in the reaction plate, create and add a detector to the plate document:

   a. Click **Add Detector**, then look for the detector that represents the target sequence in your HRM reactions and the HRM dye that you are using to detect the variants.

b. If the appropriate detector is not listed, click **New**, then complete the Add Detector dialog box:
   - Name: Enter a name for the detector to identify the target sequence
   - Reporter: **MeltDoctor**
   - Quencher: **Non Fluorescent**

   Note: SDS Software uses the term *detector*; the HRM software uses the term *target*. When completing the Add Detector dialog box, note that the name you assign to the detector will appear as the target name in the HRM software.

c. Select the appropriate detector, click **Copy To Plate Document**, then click **Done**.

d. If not already selected, select the **Setup** tab to view the Well Inspector.

e. In the plate grid, select the wells that contain the detector, including negative controls, positive controls, and sample reactions, then select the **Use** checkbox for the detector that is in those wells.

Note: If you are running multiple assays, make sure you create and add a detector for each target sequence in the plate. The HRM Software will automatically separate the wells into different assays according to the detector assigned to the well.

3. Set up the file to match the layout of your reaction plate:
   - To indicate which wells in the reaction plate contain negative controls, select the negative control wells in the plate grid, then select **NTC** from the Task dropdown menu.

     Note: Negative controls are needed to control for the amplification, but they are not appropriate for HRM analysis. Make sure you select the NTC task for negative controls so that the HRM Software will automatically omit the negative controls from the HRM analysis.

   - Add sample names. For each sample, select wells in the plate grid then enter a name for the sample.

4. Set the Passive Reference to None: Select the wells in the plate grid, then select **None** from the Passive Reference dropdown menu.

Run the reaction plate to amplify the DNA

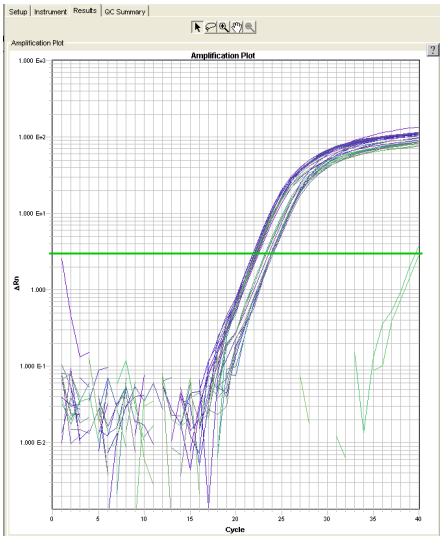
1. Set the thermal cycler protocol on the Thermal Profile and Ramp Rate tabs:
   - Mode: **Standard**
   - Sample Volume (µL):
     - **20** µL for Fast 96-well plates
     - **50** µL for standard 96-well plates
   - Thermal profile:

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%

2. Select the **Real-Time** tab, load the reaction plate into the instrument, then start the run.
3. At the prompt, browse to the location where to save the file, enter a name for your experiment, then click **Save**.
4. When the Run Complete dialog box opens, click **OK**.
5. Save the file, then unload the reaction plate.

Verify that the samples amplified

1. Click (**Analyze**), then select the **Results** tab.
2. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: If the Amplification Plot looks abnormal, refer to Chapter 6, "Troubleshooting HRM Experiments" on page 115 to identify and resolve the problem

3. Look for outliers with C_T values that differ from replicates by more than 2.

Note: Note which wells are outliers. The outliers may produce erroneous HRM results.

4. Save and close the file.

Create and set up a new run file for the melt curve

1. In the SDS Software v2.3, create a new run file for the melt curve:
   - Assay: **Standard Curve (AQ)**
   - Container: **96 Wells Clear Plate**
   - Template: **Blank Template**
   - (Optional) Scan or enter the barcode
2. Set up the file to match the layout of your reaction plate, as you did for the amplification run on page 44.

Run the reaction plate to melt the DNA

Run the reaction plate a second time to melt the DNA.

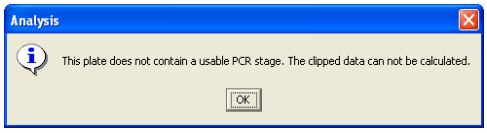
1. Spin the reaction plate briefly.
2. Set the thermal cycler protocol on the Thermal Profile and Ramp Rate tabs:
   - Mode: **Standard**
   - Sample Volume (µL):
     - **20** µL for Fast 96-well plates
     - **50** µL for standard 96-well plates
   - Thermal profile:

Stage	Step	Temp	Time	Ramp rate
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%

3. Select the **Real-Time** tab, load the reaction plate into the instrument, then start the run.
4. At the prompt, browse to the location where to save the file, enter a name for your experiment, then click **Save**.
5. When the Run Complete dialog box opens, click **OK**.
6. Save the file, then unload the reaction plate.

Review the Tm peaks

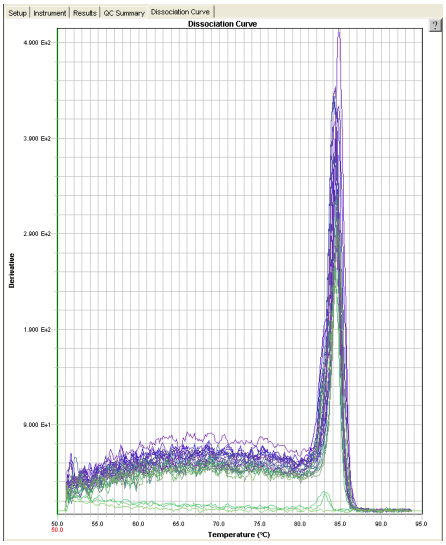
1. Click  (**Analyze**).
2. If you receive the following message, click **OK**.



3. Verify that the Dissociation Curve shows no unexpected Tm peaks.

   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 Tm peak because of the large differences between the different sequence types.

   Note: Unexpected peaks at low temperatures may indicate contamination, primer dimers, or non-specific amplification.



4. Save and close the file.

5. Proceed with "Review the high-resolution melting data" on page 56.

Run a 96-well plate on a 7500 Fast instrument with 7500 Software v2.0

Create and set up a new experiment file for the HRM run

1. In the 7500 Software, create a new experiment to amplify and melt the DNA, then set up the file in the Experiment Properties screen:
   - Experiment Name – Enter a unique name for your experiment
   - Instrument – **7500 Fast (96 Wells)**
   - Experiment type – **Quantitation - Standard Curve**
   - Reagents – **Other**, then select the **Include Melt Curve** checkbox
   - Ramp speed – **Standard (~ 2 hours to complete a run)**

2. Define each target sequence and each sample in the reaction plate in the Plate Setup ▸ Define Targets and Samples tab:
   - For each target sequence in the reaction plate, add a corresponding target to the experiment: Click the **Target Name** cell, enter a target name, then select **MeltDoctor** from the Reporter dropdown menu
   - For each sample in the reaction plate, add a sample name: Click **Add New Sample**, then enter a sample name.

3. Assign targets and samples to wells in the plate grid to match the layout of your reaction plate in the Plate Setup ▸ Assign Targets and Samples tab:
   - Set up the negative controls: Select the negative control wells in the plate grid, then select the **Assign** checkbox next to your target and select the N (**Negative Control**) task.

     Note: Negative controls are needed to control for the amplification, but they are not appropriate for HRM analysis. Make sure you assign the Negative Control task for negative controls so that the HRM Software will automatically omit the negative controls from the HRM analysis.

   - Set up the unknowns: Select the wells containing DNA samples in the plate grid, then select the **Assign** checkbox next to your target. The U Unknown task is selected by default.
   - For each sample, select the wells that contain a sample, then select the **Assign** checkbox next to the corresponding sample name.

   Note: If you are running multiple assays, make sure you define each target sequence in the plate and assign the targets to the appropriate wells. The HRM Software will automatically separate the wells into different assays according to the target assigned to the well.

4. Set the Passive Reference to **None**.

5. In the Run Method screen, set the thermal cycler conditions:
   - Reaction Volume Per Well: **20** µL
   - Thermal profile:

Stage	Step	Temp	Time
Holding	Enzyme activation	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	15 sec
	Anneal/extend	60 °C	1 min
Melt curve/dissociation	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melting	95 °C	15 sec
	Anneal	60 °C	15 sec

   - Expert Mode: Select the checkbox
   - Click **Select/View Filters**, then select only **Filter-1**

Run the plate to amplify and melt the DNA

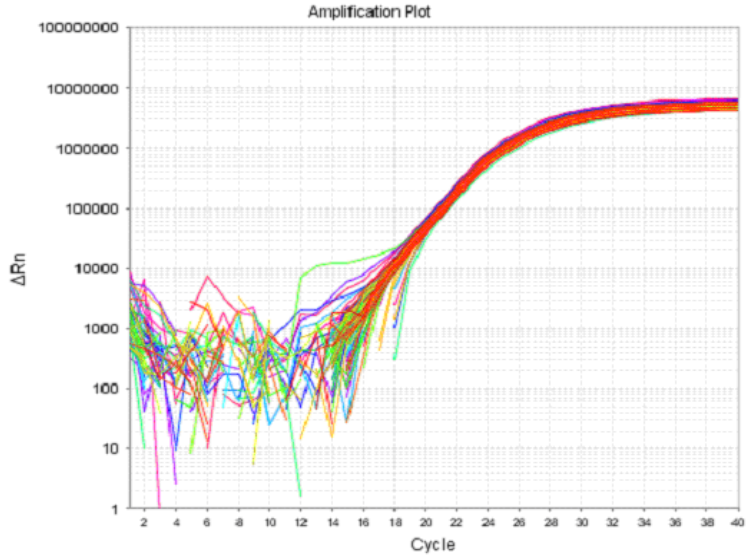
1. In the navigation pane, select **Run**, then load the reaction plate into the instrument, then click **START RUN**.

Note: You may receive a message recommending SYBR® Green reagents for melt curve experiments. Click **OK** to close the message.

2. At the prompt, save the file to a desired save location.

Verify that the samples amplified and review the Tm peaks

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: If the Amplification Plot looks abnormal, refer to Chapter 6, "Troubleshooting HRM Experiments" on page 115 to identify and resolve the problem.

2. Look for outliers with C_T values that differ from replicates by more than 2.

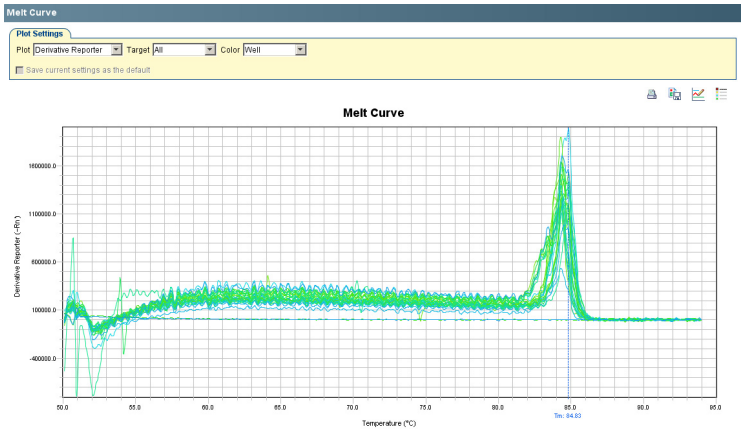
Note: Note which wells are outliers. The outliers may produce erroneous HRM results.

3. Verify that the Melt Curve shows no unexpected Tm peaks.

   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 Tm peak because of the large differences between the different sequence types.

   Note: Unexpected peaks at low temperatures may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data appear noisy because the single filter read in Expert Mode (Filter 1) results in an increase in data collection. The extra data are required for analysis with the High Resolution Melting Software.



4. Save and close the file.

5. Proceed with "Review the high-resolution melting data" on page 56.

Run a 96-well plate on a 7500 Fast instrument with SDS Software v1.4

Create and set up a new run file for the HRM run

1. In the SDS Software, create a new run file for the amplification:
   - Assay: **Standard Curve (Absolute Quantitation)**
   - Container: **96-Well Clear**
   - Template: **Blank Document**

2. For each target sequence in the reaction plate, create and add a detector to the plate document:

   a. Enter a name for the detector, select **MeltDoctor** for the Reporter Dye, then click **OK**.

   Note: SDS Software uses the term *detector*; the HRM software uses the term *target*. When completing the New Detector dialog box, note that the name you assign to the detector will appear as the target name in the HRM software.

b. Select the detector from the list, then click **Add**.

Note: If you are running multiple assays, make sure you create and add a detector for each target sequence in the plate.

3. Set up the plate grid to match the layout of your reaction plate:

   a. To indicate which detector is in being used in each well, select the wells that contain a particular detector, including negative controls, positive controls, and sample reactions, then select the **Use** checkbox for the corresponding detector.

   Note: If you are running multiple assays, make sure you indicate the detector used in each well. The HRM Software will automatically separate the wells into different assays according to the detector assigned to the well.

   b. To indicate which wells in the reaction plate contain negative controls, select the negative control wells in the plate grid, then select **NTC** from the Task dropdown menu.

   Note: Negative controls are needed to control for the amplification, but they are not appropriate for HRM analysis. Make sure you select the NTC task for negative controls so that the HRM Software will automatically omit the negative controls from the HRM analysis.

   c. Add sample names. For each sample, select wells in the plate grid then enter a name for the sample.

   d. From the Passive Reference dropdown menu, select **(none)**.

Run the plate to amplify and melt the DNA

1. Set the thermal cycler protocol in the **Instrument ▸ Thermal Profile** tab:
   - Thermal profile:

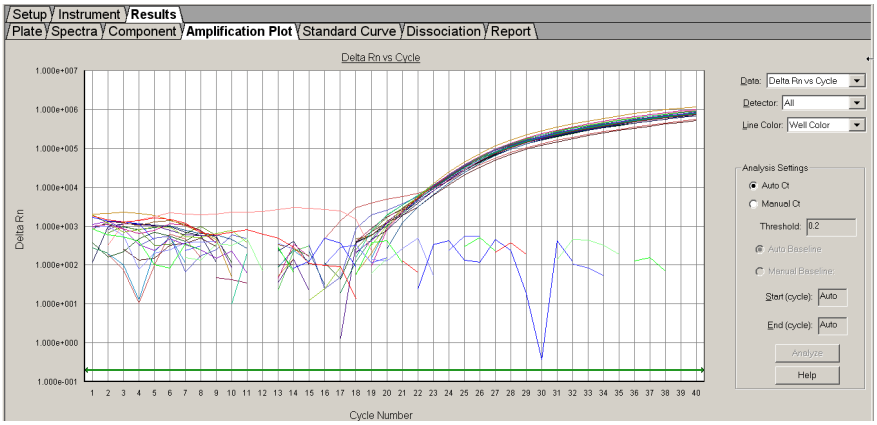
Stage	Step	Temp	Time
Holding	Enzyme activation	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	15 sec
	Anneal/extend	60 °C	1 min
Melt curve/dissociation	Denature	95 °C	10 sec
	Anneal	60 °C	1 min
	High resolution melting	95 °C	15 sec
	Anneal	60 °C	15 sec

   - Sample Volume (µL): **20**
   - Run Mode: **Fast 7500**
   - Expert Mode: Select the checkbox
   - Click **Select/View Filters**, then select only **Filter A**
2. Load the reaction plate into the instrument, then start the run.
3. At the prompt, browse to the location where to save the file, enter a name for your experiment, then click **Save**.
4. When the *Run completed successfully* message appears, click **OK**.
5. Save the file, then unload the reaction plate.

Verify that the samples amplified and review the Tm peaks

1. Click (**Analyze**), then select the **Results** tab.

2. View the Amplification Plot, then review the plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: If the Amplification Plot looks abnormal, refer to Chapter 6, "Troubleshooting HRM Experiments" on page 115 to identify and resolve the problem.

3. Look for outliers with C_T values that differ from replicates by more than 2.

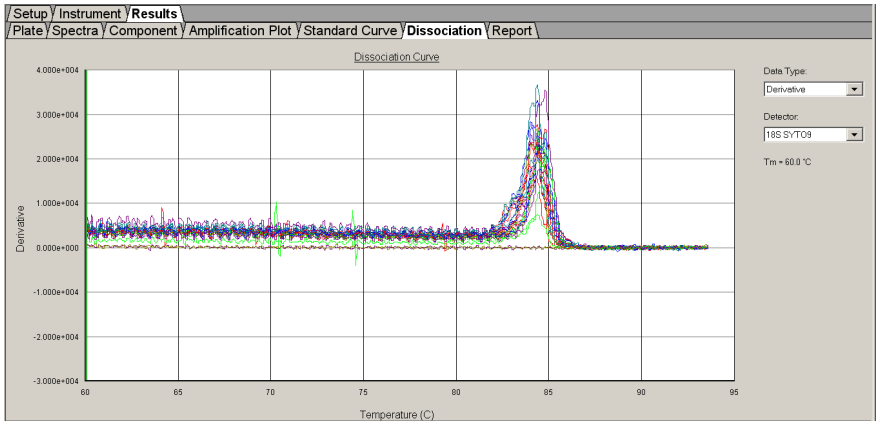
Note: Note which wells are outliers. The outliers may produce erroneous HRM results.

4. View the Dissociation Curve, then verify that the curve shows no unexpected Tm peaks.

   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 Tm peak.

   Note: Unexpected peaks may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data appear noisy because the single filter read in Expert Mode (Filter A) results in an increase in data collection. The extra data are required for analysis with the High Resolution Melting Software.



5. Save and close the file.

6. Proceed with "Review the high-resolution melting data" on page 56.

Review the high-resolution melting data

After you create, run, and analyze the *.eds or *.sds file on the 7900HT Fast or 7500 Fast system, use the Applied Biosystems High Resolution Melting Software (HRM software) to perform high resolution melting analysis of the data and review the variants.

Start the HRM Software

Start the HRM Software:

- On the desktop, double-click  (HRM v2.0 or later)

  or

- Select **Start** ▸ **All Programs** ▸ **Applied Biosystems** ▸ **HRM** ▸ **HRMv2.0** (or later)

Access the HRM Help system

The HRM software has a Help system that describes how to use each feature of the software. Access the HRM Software Help by doing one of the following:

- Click  in the software window
- Select **Help** ▸ **HRM Help**
- Press **F1**

About the HRM calibration file

The HRM calibration file is the *.eds or *.sds file that you created, ran, and analyzed on the 7500 Fast or 7900HT Fast System during the HRM calibration (see Chapter 1).

When you create an HRM experiment file in the HRM Software, the software assigns an HRM calibration file to the HRM run file. The data from the HRM calibration file are used in the HRM analysis.

The first time that you create an HRM experiment file in the HRM Software, select the default HRM calibration file to assign to HRM experiment files.

Example HRM experiments

To view an example of an HRM experiment, use the example files that are installed with the HRM software:

- 384well Genotyping Example.hrm
- 96well Genotyping Example.hrm

The files are located in *X*:\Applied Biosystems\HRM\experiments, where *X* is the drive where you installed the HRM Software.

Create the HRM experiment

Create an HRM experiment in the HRM Software using the *.eds or *.sds run file from your 7900HT Fast or 7500 Fast system.

The HRM software uses the default analysis settings to automatically assign a variant call to each sample. The software determines the variant calls by melt curve characteristics – melt curve shapes and Tm values. Before you assign the controls, the software labels each variant call *variant1*, *variant2*, *variant3*, and so on.

1. Using the HRM Software, select **File ▸ Open** from the menu bar *or* click 

in the toolbar.

2. Browse to and select the *.eds or *.sds file to undergo HRM analysis.

3. If this is your first time to open an HRM run file, the Open Calibration File dialog box opens. Browse to and select the HRM calibration file to use as the default HRM calibration file.

   IMPORTANT! Use the HRM calibration file name to make sure that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

   - Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
   - Run using the same HRM dye and master mix used in the HRM calibration plate

   The HRM Software cannot distinguish between all calibration files. For example, it cannot distinguish between two different 7500 v2.0 instruments or between a 7900HT instrument with the Fast 96-well block and a 7900HT instrument with a standard 96-well block.

   Note: If you cannot see the Browse button, resize the dialog box.

   To change the HRM calibration file for a selected experiment or to change the default HRM calibration file for subsequent new HRM experiments, see "Change the HRM calibration file" on page 131.

4. Click **OK**.

   A new HRM experiment appears in the HRM Experiments pane. The default name is the name of the *.eds or *.sds file you opened in step 2.

   Note: An HRM experiment is an *.hrm file that contains one run file and one HRM calibration file. Each HRM experiment in the software appears as a folder icon  in the HRM Experiments pane; expand the folder to view the HRM run file and HRM calibration file names.

5. Save the new HRM experiment:

   a. In the HRM Experiments pane, select the new HRM experiment.

   b. In the toolbar, click 

.

   c. Browse to and select a save location, then accept the default name or enter a new name for the HRM experiment.

d. Click **Save**.

The new HRM experiment is saved as an *.hrm file.

Note: You cannot open a run file or an HRM experiment by double-clicking the file icon (*.eds, *.sds, or *.hrm file) from outside the HRM Software.

About the melting profiles

The melting profile of a PCR product depends on its GC content, length, sequence, and heterozygosity. High-resolution melting analysis calls variants based on the differences in the shape of the melt curves and the differences in the Tm values.

With the MeltDoctor™ HRM Positive Control Kit:

- The heterozygotes have a different curve shape compared to the wild type homozygote and the variant homozygote. The shape of the melt curve is an indicator of heteroduplex formation.
- The two homozygotes are distinguished from each other based on the difference in Tm values.

About the pre- and post-melt regions

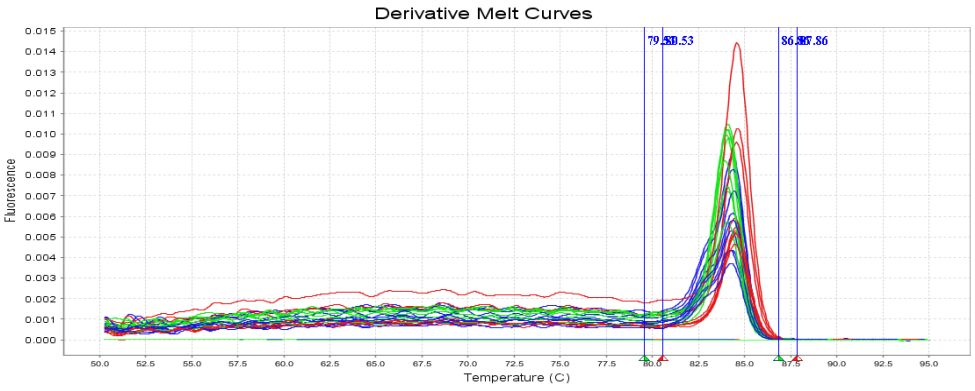
In the Derivative Melt Curves plot and the Raw Melt Curves plot, there are two sets of lines before and after the data peak. The pre- and post-melt regions are used to scale the data in the Aligned Melt Curves and Difference Plot.

- Pre-melt region: The set of lines to the left of the peak indicate the pre-melt Start and Stop temperatures when every amplicon is double-stranded.

  Fluorescence data from the pre-melt region correspond to 100% fluorescence.
- Active melt region: The data peak indicates the active melt region of the plot. For each sample, the change in fluorescence to the right of the 100% fluorescence point correspond to the true fluorescence change. Data from the active melt region are used to plot the Aligned Melt Curves.

  Note: In methylation studies, the Derivative Melt Curves plot typically displays multiple peaks.
- Post-melt region: The set of lines to the right of the peak indicate the post-melt Start and Stop temperatures when every amplicon is single-stranded.

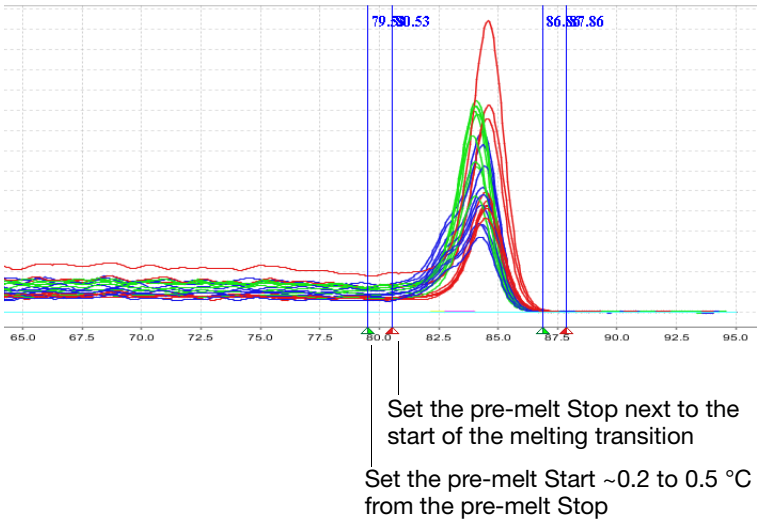
  Fluorescence data from the post-melt region correspond to 0% fluorescence.



Review the pre- and post-melt regions

When you create a new HRM experiment, the software calculates the pre- and post-melt regions automatically. Review and adjust the pre- and post-melt regions to optimize your separation and variant calls. For most experiments, set the pre- and post-melt regions as close as possible to the melting transition region.

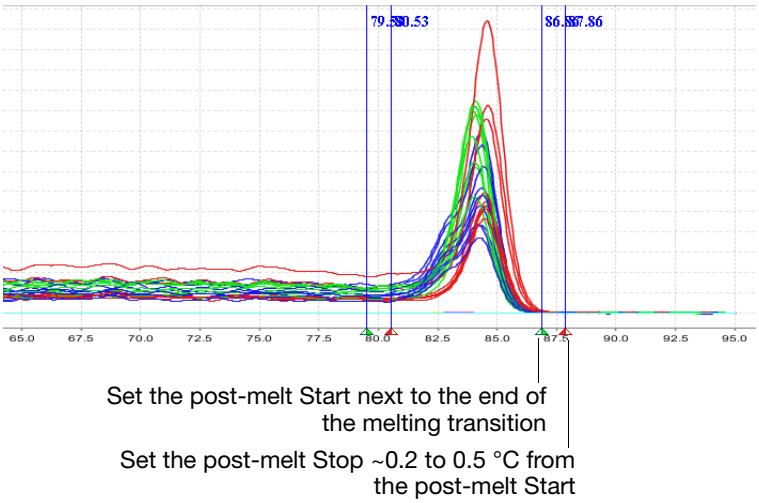
1. In the Data pane, select the **Derivative Melt Curves** tab.
2. Press **Ctrl+A** to select all the wells.
3. Set the pre-melt region:
   a. Click and drag the pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melting transition region.
   b. Click and drag the pre-melt Start temperature line (green arrow on the left) approximately 0.2 to 0.5 °C from the pre-melt Stop temperature line.



Note: The pre-melt region should be within a flat area where there are no large spikes or slopes in the fluorescent levels.

4. Set the post-melt region:
   a. Click and drag the post-melt Start temperature line (green arrow on the right) adjacent to the end of the melting transition region.

b. Click and drag the post-melt Stop temperature line (red arrow on the right) approximately 0.2 to 0.5 °C from the post-melt Start temperature line.



Note: The post-melt region should be within a flat area where there are no large spikes or slopes in the fluorescent levels.

5. Click Analyze.

   The software reanalyzes the data using the new pre- and post-melt regions. In both the Analyzed Data and Data panes, the color of the melt curves changes to reflect the new results.

Assign controls

For each positive control sample, enter information about that control in the HRM software and assign the control to the appropriate wells.

Note: Negative control samples that were designated as NTCs or Negative Controls using the instrument software are automatically omitted from analysis in the HRM Software.

1. In the toolbar, click Assign Control.
2. On the Assign Controls tab, click **Add**.
3. In the Control Name field, enter a name for a control sample.

   Note: For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The HRM software uses the convention *variantN* when automatically assigning the variant calls.

4. From the Well dropdown list, select the well that contains the sample to use as the control.

5. Select the color to display in the plots for this control.

6. Repeat step 2 through step 5 to assign the remaining controls.

Note: For control replicates, enter a name and select a color *identical* to the other control replicates. You can enter up to 5 replicates for each control.

The example experiment file, 384well Genotyping Example.hrm, contains the following controls:

Control Name	Well	Color
Hom - wild type	A4	
Hom - wild type	P18	
Hom - variant	A3	
Hom - variant	P21	
Heterozygote	A2	
Heterozygote	E22	

7. Click **Analyze** to reanalyze the data with the controls assigned.

Note: In the Results pane, the variant call for the sample(s) you selected is renamed using the convention Control-<*name*>, where <*name*> is the name you entered in step 3.

Review the populations in the Aligned Melt Curves plot

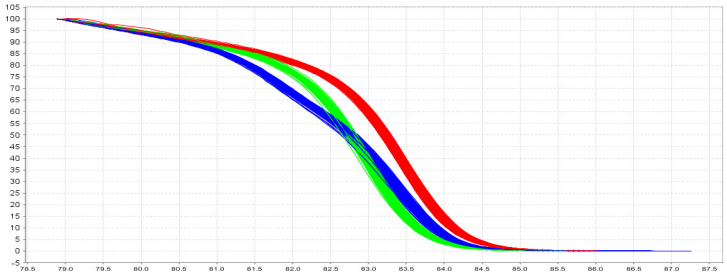
The Aligned Melt Curves plot displays the melt curves as % melt (0 - 100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set (see page 59).

1. In the Analyzed Data pane, select the **Aligned Melt Curves** tab.

2. Review:
   - Variant groups (different colors) – How many different variant groups are displayed? Does this number correspond to the number of variants you were expecting?
   - Outliers – Are there any curves within a variant group that do not cluster tightly with the other samples in that group?

Aligned Melt Curves example

In the example below, there are 3 distinct variant groups, 1 for each genotype. The wild type control (homozygote) is selected as the reference (green curves).



Review the Difference Plot for outliers

The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

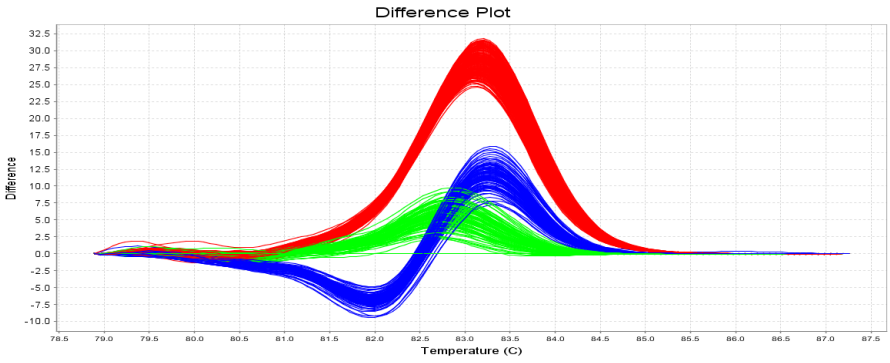
The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the Analyzed Data pane, select the **Aligned Data - Difference Plot** tab.
2. From the **Reference** dropdown menu, select a control or any well as the reference, then review:
   - Variant clusters – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant cluster?

Note: Try selecting different reference samples to find the optimal display of the clusters.

Difference Plot example

In the example below, there are 3 distinct variant groups, 1 for each genotype. The wild type control (homozygote) is selected as the reference (green curves).



Review the software calls

The High Resolution Melting Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.

2. For the positive controls, review:
   - Variant Call column – Do all of the positive control replicates have the correct call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.

3. For each replicate group, review:
   - Variant Call column – Do all replicates have the same call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?

4. To view the fluorescence data for certain wells, select the rows in the Results table.

5. In the Results pane, click the **Variant Call** column header to sort the results according to the variant call. For each variant call, review the samples that were assigned that call.

Omit outliers from analysis

After you review the data, omit outliers from the analysis. You can omit outliers in the Data pane, Analyzed Data pane, or Results pane.

Note: Try omitting outliers from the Difference Plot, where the variance is easily visualized.

1. Select the outliers, then omit the wells:

Pane	Procedure
Data or Analyzed Data pane	1. Click and drag in the Raw Melt Curves, Derivative Melt Curves, Aligned Melt Curves, or Difference Plot to create a box and select one or more outlier curves. The color of the selected curves changes. 2. Right-click, then select **Omit Wells**.
Results pane	1. Select the Omit checkbox for any well to omit from the HRM analysis.

2. Click 

. The software omits data from the selected wells and reanalyzes the remaining data.

Change calls made by the software

If you do not agree with the call automatically made by the software (Auto call), you can manually change the call in the Analyzed Data pane or the Results pane.

1. Select samples, then manually change the call:

Pane	Procedure
Analyzed Data pane	1. Click and drag in the Aligned Melt Curves or Difference Plot to create a box and select one or more curves with a call to change. The color of the selected curves changes. 2. Right-click, then select **Manual call**. To change the call to: • A call that has already been made for other samples: Select the appropriate call from the dropdown menu. • A new call: Click **New**, enter a name for the new call, click **OK**, select a color, then click **OK**.
Results pane	1. Click the cell in the **Variant Call** column for the call to change. 2. From the dropdown menu, you can change the call to: • A call that has already been made for other samples: Select the appropriate call from the dropdown menu. • A new call: Select **Manual call**. In the dialog box, enter a name for the new call, select a color, then click **OK**. In the Comments column, *Manual call* appears next to the sample.

2. Click 

. The software reanalyzes the data using your manual calls.

Revert selected manual calls to the software Auto call

If you want to remove a manual call for selected samples, you can revert the manual call to the call automatically made by the software (Auto call) in the Analyzed Data pane or the Results pane.

1. Select samples, then revert the manual call:

Pane	Procedure
Analyzed Data pane	1. Click and drag in the Aligned Melt Curves or Difference Plot to create a box and select one or more curves with a manual call to revert. The color of the selected curve(s) changes. 2. Right-click, then select **Auto call**.
Results pane	1. Click the cell in the **Variant Call** column for the call to revert. 2. Select **Auto call** from the dropdown menu.

2. Click 

. The software reanalyzes the data using the calls for the selected samples.

Revert all manual calls to the software Auto call

If you want to remove all manual calls for all samples in the plate, you can revert all manual calls to the call automatically made by the software (Auto call).

1. In the toolbar, select 

.

2. Select the **Remove All Manual Calls** checkbox.
3. Click 

. The software reanalyzes the data using the software Auto call for all samples.

3 Perform an HRM Genotyping Experiment

Perform an HRM genotyping experiment to determine the genotype of a DNA sample.

Perform an HRM Genotyping Experiment

Design the HRM experiment (below)

↓

Prepare the HRM reactions (page 68)

↓

Amplify and melt the DNA (page 71)

↓

Review the high-resolution melting data (page 74)

Design the HRM experiment

Design and order the primers

Using Primer Express® Software v3.0 or later, design the primers to amplify the genomic DNA that contains the single nucleotide polymorphism (SNP) of interest. Order the primers from the Applied Biosystems Store.

1. Design the primers so that they meet these guidelines:

Design attribute	Design guidelines
Amplicon	• Length is less than 250 basepairs • Contains only 1 SNP
Primer length	~20 bases each
Tm	58 °C to 60 °C (Optimal Tm is 59 °C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

2. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Ordering custom primers" on page 128.

Select controls

Include controls for each SNP sequence in your HRM genotyping experiment:

- At least one negative control
- At least one positive control to represent each expected genotype

  Run 3 to 5 replicates for each expected genotype to improve your results. Running multiple positive controls allows you to more effectively define the natural spread or variation within different samples of the same sequence, or within replicates of the same genotype.

Prepare the HRM reactions

Combine purified genomic DNA templates with MeltDoctor™ HRM Master Mix and primers to amplify the target sequence.

Note: If you are using the MeltDoctor™ HRM Reagent Kit instead of the MeltDoctor™ HRM Master Mix, see page 129 for reaction component volumes.

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your Real-Time PCR instrument
- MicroAmp™ Optical Adhesive Film
- MeltDoctor™ HRM Master Mix
- For each target sequence:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Prepare the HRM reactions

Prepare the reactions for each replicate group separately, then transfer the reactions to a reaction plate appropriate for your instrument.

For information about using the MeltDoctor™ HRM Reagent Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 129.

1. Prepare the negative control reactions in an appropriately sized, labeled tube:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Deionized water	7.6 µL	25.08 µL	19.0 µL	62.7 µL
Total reaction volume	**20.0 µL**	**66.00 µL**	**50.0 µL**	**165.0 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

2. Prepare positive control reactions and unknown reactions in separate appropriately sized, labeled tubes:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL	2.5 µL	8.25 µL
Deionized water	6.6 µL	21.78 µL	16.5 µL	54.45
Total reaction volume	**20 µL**	**66 µL**	**50 µL**	**165 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

3. Vortex the reactions to mix, then spin the tubes briefly.

4. Prepare a reaction plate appropriate for your instrument:

   a. Pipet each reaction replicate to the appropriate wells of the optical reaction plate:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.

   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4 °C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data. This chapter contains brief instructions. For detailed instructions, see:

- Run a 384-well plate on a 7900HT Fast instrument (page 40)
- Run a 96-well plate on a 7900HT Fast instrument (page 43)
- Run a 96-well plate on a 7500 Fast instrument with 7500 Software v2.0 (page 48)
- Run a 96-well plate on a 7500 Fast instrument with SDS Software v1.4 (page 51)

Create and set up the HRM run file

Run file setting	7900HT Fast System with SDS Software v2.3 or later	7500 Fast System with 7500 Software v2.0 or later	7500 Fast Real-Time PCR System with SDS Software v1.4
Document/ experiment	• Assay: **Standard Curve (AQ)** • Container: **384 Wells Clear Plate** or **96 Wells Clear Plate** • Template: **Blank Template**	• Instrument: **7500 Fast (96 Wells)** • Experiment type: **Quantitation - Standard Curve** • Reagents: **Other**, then select the **Include Melt Curve** checkbox • Ramp speed: **Standard (~ 2 hours to complete a run)**	• Assay: **Standard Curve (Absolute Quantitation)** • Container: **96-Well Clear** • Template: **Blank Document**
Detector/target and plate layout	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**	• Reporter: **MeltDoctor** • Quencher: **None**	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**
Plate layout	• Task for negative control wells: **NTC** • Passive Reference: **None**	• Task for negative control wells: N • Passive Reference: **None**	• Task for negative control wells: **NTC** • Passive Reference: **(none)**
Thermal profile/run method	• Mode: **Standard** • Sample Volume (µL): **20** (384-well or 96-well Fast) or **50** (96-well standard)	• Reaction Volume Per Well: **20** µL • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter-1**	• Sample Volume (µL): **20** • Run Mode: **Fast 7500** • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter A**

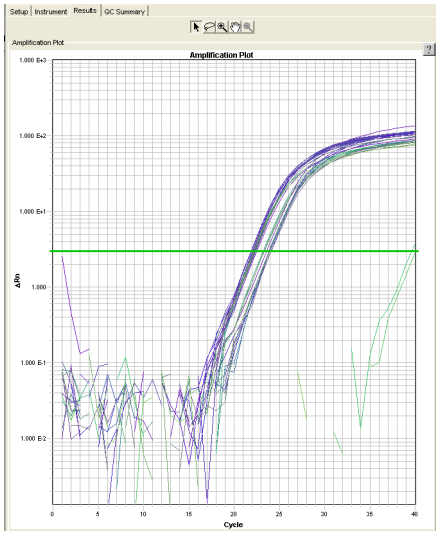
Run the plate

Note: If you are performing your experiment on the 7900HT Fast instrument using a 96-well reaction plate, perform the melt curve in a separate run because you need to spin the plate after you amplify the DNA.

Stage	Step	Temp	Time	Ramp rate (7900HT only)
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%

Verify that the samples amplified and review the peaks in the melt curve

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: Note which wells are outlierswith C_T values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

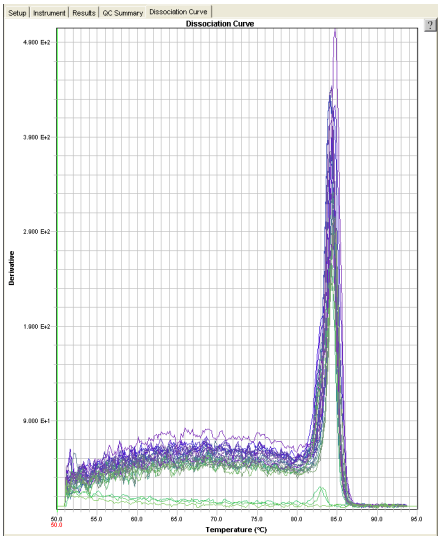
Note: If the Amplification Plot looks abnormal, refer to Chapter 6, "Troubleshooting HRM Experiments" on page 115 to identify and resolve the problem.

2. Verify that the Dissociation Curve/Melt Curve shows no unexpected Tm peaks:

   If the sequence you amplified contains more than 1 SNP or a more complex mutation, you may see more than 1 Tm peak.

   Note: Unexpected peaks may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the High Resolution Melting Software.



Review the high-resolution melting data

After you create, run, and analyze the *.eds or *.sds file on the 7900HT Fast or 7500 Fast system, use the Applied Biosystems High Resolution Melting Software (HRM software) to perform high resolution melting analysis of the data and make genotype calls.

Example HRM experiments

To view an example of an HRM genotyping experiment, use the example files that are installed with the HRM software:

- 384well Genotyping Example.hrm
- 96well Genotyping Example.hrm
- 96well Class 4 SNP Example.hrm

The files are located in *X*:\Applied Biosystems\HRM\experiments, where *X* is the drive where you installed the HRM Software.

Create and set up the HRM experiment

For more detailed instructions on how to create and set up an HRM experiment, see pages 56 through 60.

1. Create an HRM experiment in the HRM Software using the *.eds or *.sds run file from your 7900HT Fast or 7500 Fast system.

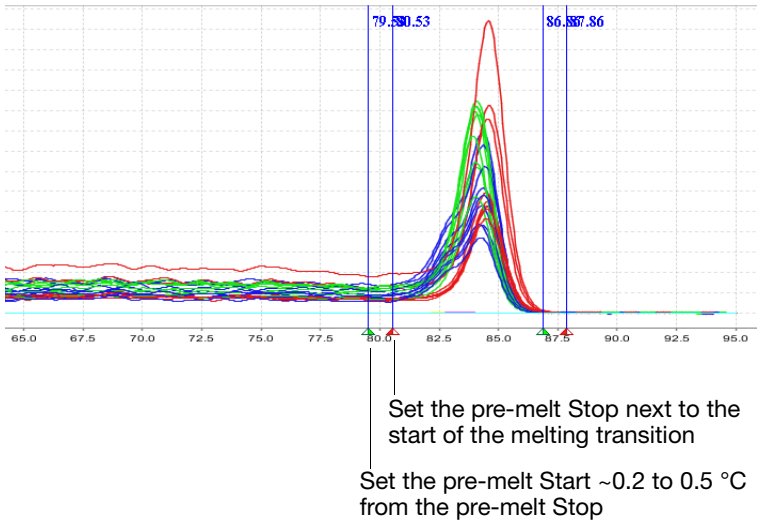
Note: If this is your first time creating an HRM experiment with the HRM software, select the default HRM calibration file. To change the HRM calibration file for a selected experiment or for all subsequent HRM experiments, see "Change the HRM calibration file" on page 131.

IMPORTANT! Use the HRM calibration file name to make sure that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

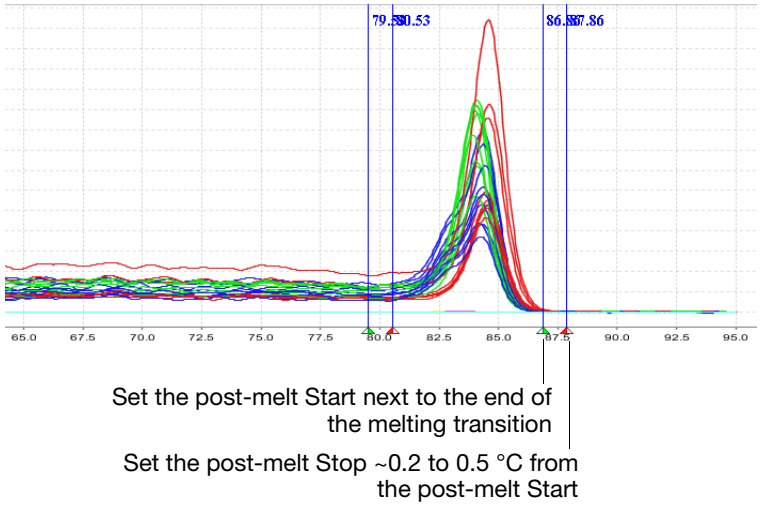
- Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
- Run using the same HRM dye and master mix used in the HRM calibration plate

The HRM Software cannot distinguish between all calibration files. For example, it cannot distinguish between two different 7500 v2.0 instruments or between a 7900HT instrument with the Fast 96-well block and a 7900HT instrument with a standard 96-well block.

2. In the Derivative Melt Curves plot, review and adjust the pre- and post-melt regions to optimize your separation and variant calls. For most experiments, set the pre- and post-melt regions as close as possible to the melting transition region:
   - The pre-melt Start and Stop temperature lines (green and red arrows on the left) should be approximately 0.2 to 0.5 °C apart from each other.



   - The post-melt Start and Stop temperature lines (green and red arrows on the right) should be approximately 0.2 to 0.5 °C apart from each other.



3. For each control sample, enter information about that control in the HRM Software and assign the control to the appropriate wells.

   The example experiment file, 384well Genotyping Example.hrm, contains the following controls:

Control Name	Well	Color
Hom - wild type	A4	
Hom - wild type	P18	
Hom - variant	A3	
Hom - variant	P21	
Heterozygote	A2	
Heterozygote	E22	

Note: For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The HRM Software uses the convention *variantN* when automatically assigning the variant calls.

About the melting profiles

The melting profile of a PCR product depends on its GC content, length, sequence, and heterozygosity. High-resolution melting analysis calls variants based on the differences in the shape of the melt curves and the differences in the Tm values.

In genotyping experiments:

- The heterozygotes have a different curve shape compared to the wild type homozygote and the variant homozygote. The shape of the melt curve is an indicator of heteroduplex formation.
- The two homozygotes are distinguished from each other based on the difference in Tm values.

Review the populations in the Aligned Melt Curves plot

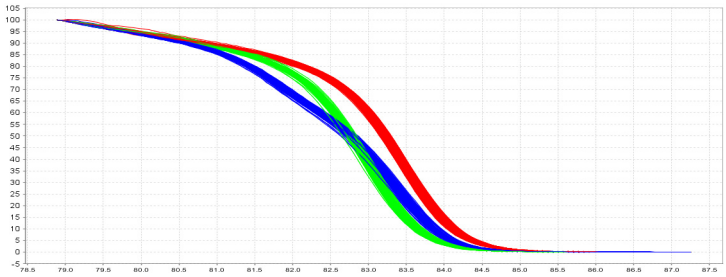
The Aligned Melt Curves plot displays the melt curves as % melt (0 - 100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set (see page 74).

1. In the Analyzed Data pane, select the **Aligned Melt Curves** tab.

2. Review:
   - Variant groups (different colors) – How many different variant groups are displayed? Does this number correspond to the number of variants you were expecting?
   - Outliers – Are there any curves within a variant group that do not cluster tightly with the other samples in that group?

Aligned Melt Curves example

In the example below, there are 3 distinct variant groups, 1 for each genotype. The wild type control (homozygote) is selected as the reference (green curves).



Review the Difference Plot for outliers

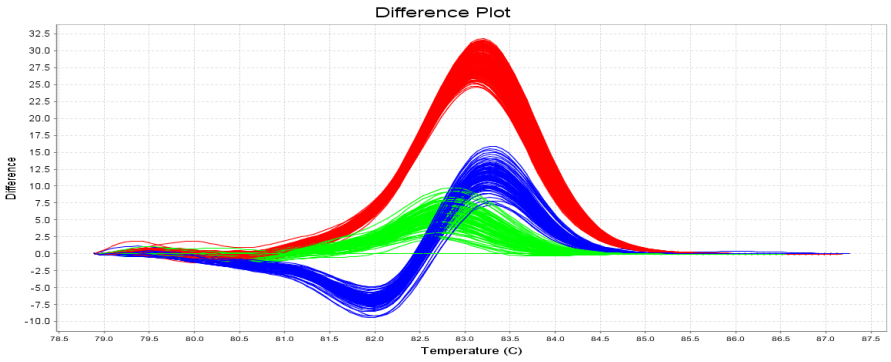
The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the Analyzed Data pane, select the **Aligned Data - Difference Plot** tab.
2. From the **Reference** dropdown menu, select the wild type control as the reference, then review:
   - Variant clusters – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant cluster?

Difference Plot example

In the example below, there are 3 distinct variant groups, 1 for each genotype. The wild type control (homozygote) is selected as the reference (green curves).



Review the software calls

The High Resolution Melting Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.

2. For the positive controls, review:
   - Variant Call column – Do all of the positive control replicates have the correct call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.

3. For each replicate group, review:
   - Variant Call column – Do all replicates have the same call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?

4. To view the fluorescence data for certain wells, select the rows in the Results table.

5. In the Results pane, click the **Variant Call** column header to sort the results according to the variant call. For each variant call, review the samples that were assigned that call.

Omit outliers or change calls

After you review the software calls, you can omit outliers or change calls. Remember to click 

to reanalyze the data after you omit outliers or change calls.

For more detailed instructions, see pages 63 and 64:

- Omit outliers from analysis
- Change calls made by the software
- Revert selected manual calls to the software Auto call
- Revert all manual calls to the software Auto call

4 Perform an HRM Mutation Scanning Experiment

Perform an HRM mutation scanning experiment to screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations.

Perform an HRM Mutation Scanning Experiment

Design the HRM experiment (below)

↓

Prepare the HRM reactions (page 80)

↓

Amplify and melt the DNA (page 83)

↓

Review the high-resolution melting data (page 86)

↓

Sequence the variants (page 91)

Design the HRM experiment

Design and order the primers

Using Primer Express® Software v3.0 or later, design the primers to amplify the genomic DNA that spans the mutations of interest. Order the primers from the Applied Biosystems Store.

1. Design the primers so that they meet these guidelines:

Design attribute	Design guidelines
Amplicon	Length is less than 250 basepairs
Primer length	~20 bases each
Tm	58 °C to 60 °C (Optimal Tm is 59 °C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

2. If you want to use M13F and M13R primers in the sequencing reaction, add the appropriate M13 tail to the 5′ end of the primers:
   - M13F (add to the 5′ end of the forward primer): TGTAAAACGACGGCCAGT
   - M13R (add to the 5′ end of the reverse primer): CAGGAAACAGCTATGACC

3. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Ordering custom primers" on page 128.

Select controls

Include controls for each target sequence in your HRM mutation scanning experiment:

- At least one negative control
- At least one wild type control

  Run up to 5 replicates for each wild type control to improve your results. Running multiple wild type controls allows you to more effectively define the natural spread or variation within the normal population.

Prepare the HRM reactions

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your Real-Time PCR instrument
- MicroAmp™ Optical Adhesive Film
- MeltDoctor™ HRM Master Mix
- For each target sequence:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Prepare the HRM reactions

Prepare the reactions for each replicate group separately, then transfer the reactions to a reaction plate appropriate for your instrument.

For information about using the MeltDoctor™ HRM Reagent Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 129.

1. Prepare the negative control reactions in an appropriately sized, labeled tube:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Deionized water	7.6 µL	25.08 µL	19.0 µL	62.7 µL
Total reaction volume	**20.0 µL**	**66.00 µL**	**50.0 µL**	**165.0 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

2. Prepare positive control reactions and unknown reactions in separate appropriately sized, labeled tubes:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL	2.5 µL	8.25 µL
Deionized water	6.6 µL	21.78 µL	16.5 µL	54.45
Total reaction volume	**20 µL**	**66 µL**	**50 µL**	**165 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

3. Vortex the reactions to mix, then spin the tubes briefly.

4. Prepare a reaction plate appropriate for your instrument:

   a. Pipet each reaction replicate to the appropriate wells of the optical reaction plate:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.

   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4 °C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data. This chapter contains brief instructions. For detailed instructions, see:

- Run a 384-well plate on a 7900HT Fast instrument (page 40)
- Run a 96-well plate on a 7900HT Fast instrument (page 43)
- Run a 96-well plate on a 7500 Fast instrument with 7500 Software v2.0 (page 48)
- Run a 96-well plate on a 7500 Fast instrument with SDS Software v1.4 (page 51)

Create and set up the HRM run file

Run file setting	7900HT Fast System with SDS Software v2.3 or later	7500 Fast System with 7500 Software v2.0 or later	7500 Fast Real-Time PCR System with SDS Software v1.4
Document/ experiment	• Assay: **Standard Curve (AQ)** • Container: **384 Wells Clear Plate** or **96 Wells Clear Plate** • Template: **Blank Template**	• Instrument: **7500 Fast (96 Wells)** • Experiment type: **Quantitation - Standard Curve** • Reagents: **Other**, then select the **Include Melt Curve** checkbox • Ramp speed: **Standard (~ 2 hours to complete a run)**	• Assay: **Standard Curve (Absolute Quantitation)** • Container: **96-Well Clear** • Template: **Blank Document**
Detector/target and plate layout	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**	• Reporter: **MeltDoctor** • Quencher: **None**	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**
Plate layout	• Task for negative control wells: **NTC** • Passive Reference: **None**	• Task for negative control wells: N • Passive Reference: **None**	• Task for negative control wells: **NTC** • Passive Reference: **(none)**
Thermal profile/run method	• Mode: **Standard** • Sample Volume (µL): **20** (384-well or 96-well Fast) or **50** (96-well standard)	• Reaction Volume Per Well: **20** µL • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter-1**	• Sample Volume (µL): **20** • Run Mode: **Fast 7500** • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter A**

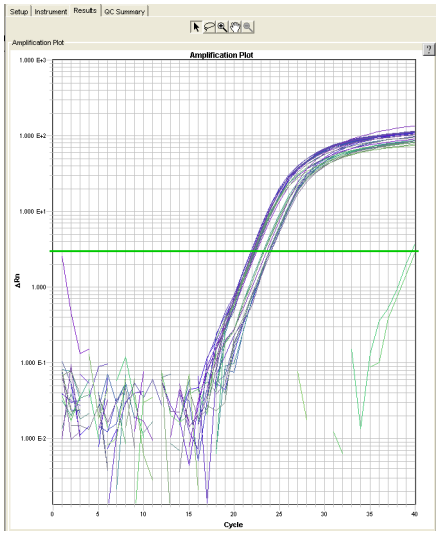
Run the plate

Note: If you are performing your experiment on the 7900HT Fast instrument using a 96-well reaction plate, perform the melt curve in a separate run because you need to spin the plate after you amplify the DNA.

Stage	Step	Temp	Time	Ramp rate (7900HT only)
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%

Verify that the samples amplified and review the peaks in the melt curve

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: Note which wells are outlierswith C_T values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

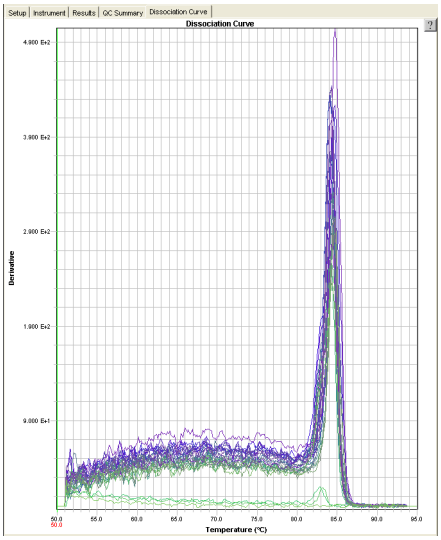
Note: If the Amplification Plot looks abnormal, refer to Chapter 6, "Troubleshooting HRM Experiments" on page 115 to identify and resolve the problem.

2. Verify that the Dissociation Curve/Melt Curve shows no unexpected Tm peaks:

   If the sequence you amplified contains more than 1 variant or a more complex mutation, you may see more than 1 Tm peak.

   Note: Unexpected peaks may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the High Resolution Melting Software.



Review the high-resolution melting data

After you create, run, and analyze the *.eds or *.sds file on the 7900HT Fast or 7500 Fast system, use the Applied Biosystems High Resolution Melting Software (HRM software) to perform high resolution melting analysis of the data and screen the samples for mutations.

Example HRM experiments

To view an example of an HRM mutation scanning experiment, use the example files that are installed with the HRM software:

- 384 Mutation Scanning Example.hrm
- 96well Mutation Scanning Example.hrm

The files are located in *X*:\Applied Biosystems\HRM\experiments, where *X* is the drive where you installed the HRM Software.

Create and set up the HRM experiment

For more detailed instructions on how to create and set up an HRM experiment, see pages 56 through 60.

1. Create an HRM experiment in the HRM Software using the *.eds or *.sds run file from your 7900HT Fast or 7500 Fast system.

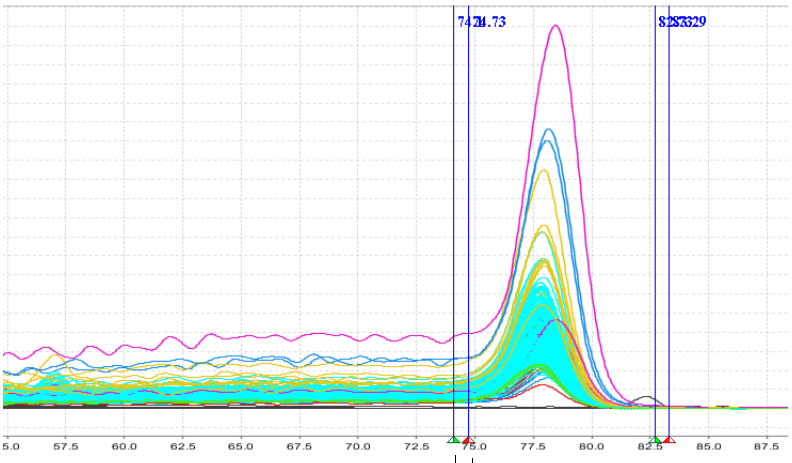
Note: If this is your first time creating an HRM experiment with the HRM software, select the default HRM calibration file. To change the HRM calibration file for a selected experiment or for all subsequent HRM experiments, see "Change the HRM calibration file" on page 131.

IMPORTANT! Use the HRM calibration file name to make sure that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

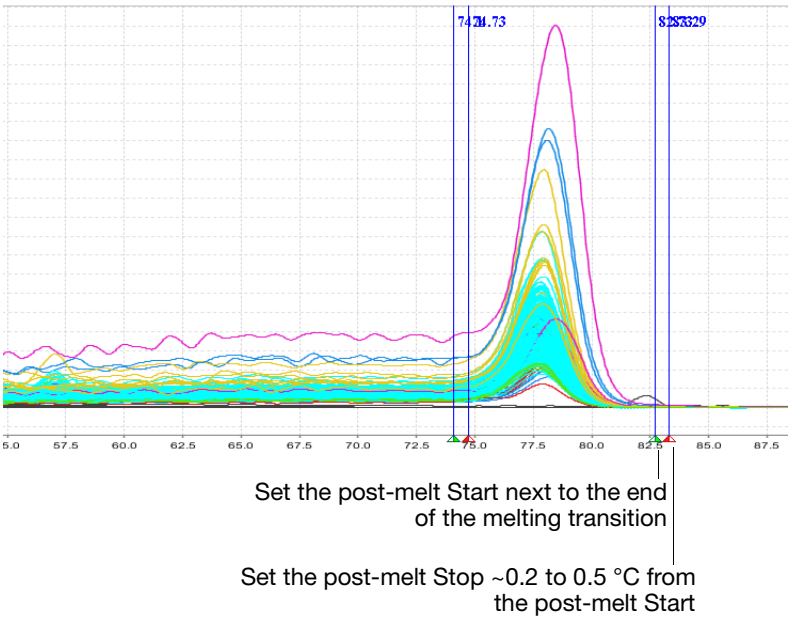
- Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
- Run using the same HRM dye and master mix used in the HRM calibration plate

The HRM Software cannot distinguish between all calibration files. For example, it cannot distinguish between two different 7500 v2.0 instruments or between a 7900HT instrument with the Fast 96-well block and a 7900HT instrument with a standard 96-well block.

2. In the Derivative Melt Curves plot, review and adjust the pre- and post-melt regions to optimize your separation and variant calls. For most experiments, set the pre- and post-melt regions as close as possible to the melting transition region:
   - The pre-melt Start and Stop temperature lines (green and red arrows on the left) should be approximately 0.2 to 0.5 °C apart from each other.



   - The post-melt Start and Stop temperature lines (green and red arrows on the right) should be approximately 0.2 to 0.5 °C apart from each other.



3. For each control sample, enter information about that control in the HRM Software and assign the control to the appropriate wells.

   The example experiment file, 384 Mutation Scanning Example.hrm, contains the following controls:

Control Name	Well	Color
Wild Type	A1	
Wild Type	G8	
Wild Type	A21	
Wild Type	E11	

Note: For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The HRM Software uses the convention *variantN* when automatically assigning the variant calls.

About the melting profiles

The melting profile of a PCR product depends on its GC content, length, sequence, and heterozygosity. High-resolution melting analysis calls variants based on the differences in the shape of the melt curves and the differences in the Tm values.

In mutation scanning experiments, the variants have a different curve shape or Tm compared to the wild type.

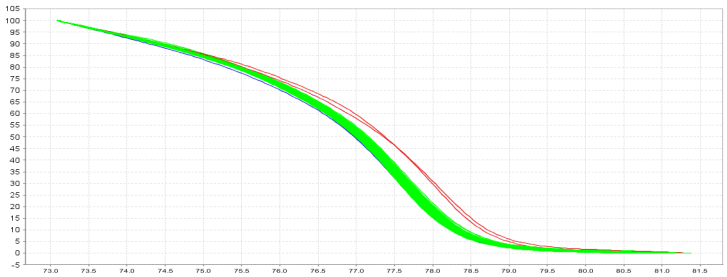
Review the populations in the Aligned Melt Curves plot

The Aligned Melt Curves plot displays the melt curves as % melt (0 - 100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set (see page 86).

1. In the Analyzed Data pane, select the **Aligned Melt Curves** tab.
2. Review:
   - Wild type controls – Do the melt curves for the wild type controls cluster well? Are there any outliers?
   - Possible mutations – Are there any samples with melt curves that are different from the wild type melt curves?

Aligned Melt Curves example

In the example below, there is 1 distinct variant group for the wild type samples. There are 2 samples that vary from the wild type samples and may contain mutations.



Review the Difference Plot for outliers

The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

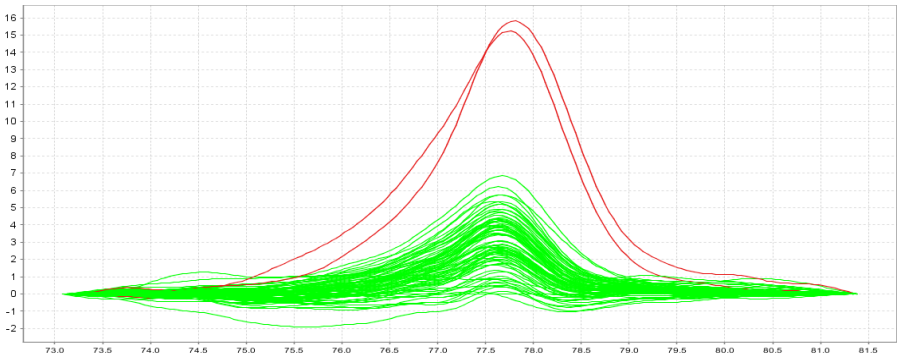
The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the Analyzed Data pane, select the **Aligned Data - Difference Plot** tab.
2. From the **Reference** dropdown menu, select a control or any well as the reference, then review:
   - Variant clusters – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant cluster?

Note: Try selecting different reference samples to find the optimal display of the clusters.

Difference Plot example

In the example below, there is 1 distinct variant group for the wild type samples. There are 2 samples that vary from the wild type samples and may contain mutations.



Review the software calls

The High Resolution Melting Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For each replicate group, review:
   - Variant Call column – Do all replicates have the same call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?
3. To view the fluorescence data for certain wells, select the rows in the Results table.

4. In the Results pane, click the **Variant Call** column header to sort the results according to the variant call. Scan the results for samples that were not assigned the same call as the wild type control.

Omit outliers or change calls

After you review the software calls, you can omit outliers or change calls. Remember to click 

to reanalyze the data after you omit outliers or change calls.

For more detailed instructions, see pages 63 and 64:

- Omit outliers from analysis
- Change calls made by the software
- Revert selected manual calls to the software Auto call
- Revert all manual calls to the software Auto call

Sequence the variants

After you identify the variants in the HRM software, dilute or purify the PCR product from the HRM reactions, then sequence the variants.

Dilute the PCR product

1. After the PCR amplification, spin the HRM reaction plate at 100 × g for 1 minute.
2. Perform DNA quantitation of the PCR products for the selected variants, then dilute to 0.5–1.5 ng/µL with water.
3. Use the dilution ratio to determine whether you need to purify the PCR product before performing the sequencing reactions:

How much did you dilute the PCR product?	Next step
<1:20	Purify the PCR product using ExoSAP-IT® (next procedure) before performing the sequencing reactions.
>1:20	Perform the sequencing reactions using the diluted DNA (page 92).

Purify the PCR product

If you diluted the PCR product less than 1:20, purify the PCR product using ExoSAP-IT®.

1. Combine the diluted PCR product and ExoSAP-IT in a clean MicroAmp® Fast Optical Reaction Plate:

Component	Volume
Diluted PCR product	10 µL
ExoSAP-IT®	2 µL
Total reaction volume	**12 µL**

2. Mix the reactions well by pipetting up and down with a multichannel pipettor, then seal the plate with MicroAmp® Clear Adhesive Film.
3. Spin the plate at 1600 × g for 30 seconds.
4. Load the plate in the thermal cycler, cover the plate with an MicroAmp® Optical Film Compression Pad, then run the reactions in a thermal cycler:
   - Reaction volume: 12 µL
   - Thermal profile:

Stage	Temp	Time
1	37 °C	30 min
2	80 °C	15 min
3	4 °C	∞

5. After the run is complete, spin the plate at 100 × g for 1 minute.

Perform the sequencing reactions

Perform fast cycle sequencing with modifications to the protocol for the BigDye® Terminator v1.1 Cycle Sequencing Kit. If your PCR products contain an M13 tail from the primers you used in the HRM amplification reactions, use the M13F and M13R primers for the forward and reverse primers.

1. On ice, prepare 8 µL of Sequencing Master Mix for each sample:

Component	Volume
BigDye® Terminator v1.1	2 µL
Forward primer or reverse primer	1 µL
Deionized water	4 µL
BigDye® Terminator v1.1, v3.1 5X Sequencing Buffer	1 µL
Total volume per reaction	**8 µL**

Note: Include 5–10% excess volume in the master mix to compensate for pipetting error.

2. Transfer 8 µL of Sequencing Master Mix to wells of a 96-well reaction plate.

3. Add 2 µL of diluted DNA to the appropriate wells of the reaction plate, then pipet up and down to mix.

4. Seal the plate with MicroAmp® Clear Adhesive Film, then spin briefly.

5. Run the reactions in a Veriti™ 96-Well Fast Thermal Cycler:
   - Reaction volume: 10 µL
   - Thermal profile:

Stage	Step	Temp	Time
Holding	Denaturation	96 °C	1 min
Cycle sequencing (25 cycles)	Denaturation	96 °C	10 sec
	Annealing	50 °C	3 sec
	Extension	60 °C	75 sec
Holding	Holding	4 °C	∞

Note: Use a rapid thermal ramp (1 °C/second) for each new temperature.

6. After the run is complete, spin the plate briefly.

Purify the sequencing reaction

Use the BigDye XTerminator® Purification Kit to remove unincorporated BigDye® terminators. For more instructions on the purification or on transferring the plate to the DNA Analyzer, refer to the *BigDye XTerminator® Purification Kit Protocol* (PN 4374408).

1. Spin the reaction plate briefly, then add BigDye XTerminator reagents:
   a. Add 45 µL of SAM™ Solution to each reaction.
   b. Vortex the BigDye XTerminator® Solution thoroughly, then use a wide-bore pipette tip to add 10 µL to each reaction.
2. Seal the plate with MicroAmp® Clear Adhesive Film, then verify that each well is sealed.
3. Vortex the plate for 30 minutes.
4. Spin the plate at 1000 × g for 2 minutes.

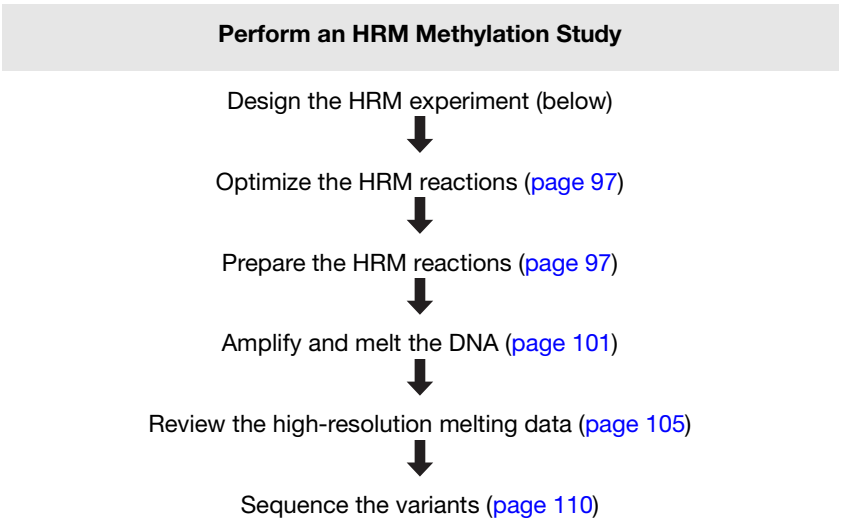
Run the sequencing reaction products

Run the sequencing reaction products on an Applied Biosystems 3500/3500*xl* DNA Analyzer, 3730/3730*xl* DNA Analyzer, 3130/3130*xl* Genetic Analyzer, or 3100/3100-*Avant* Genetic Analyzer. For more instructions, refer to the user guide for your instrument.

Item	Applied Biosystems 3500/3500*xl* DNA Analyzer with 3500 Data Collection Software v1.0	Applied Biosystems 3730/3730*xl* DNA Analyzers with Data Collection Software v1.1, v2.0, or v3.0	Applied Biosystems 3130/3130*xl* DNA Analyzer with Data Collection Software v2.0	ABI PRISM® 3100/3100-*Avant* Genetic Analyzer with Data Collection Software v2.0
Polymer	POP-7™ polymer	POP-7™ polymer	POP-6™ polymer	POP-6™ polymer
Array	50 cm	36 cm	36 cm	36 cm
Run file	RapidSeq_BDX_50_POP7	BDX_RapidSeq36_POP7	BDX_RapidSeq36_POP6	BDX_RapidSeq36_POP6
Mobility file	Kb_3500_POP6_BDV1	KB_3730_POP7_BDTv1.mob	Kb_3130_POP6_BDV1.mob	Kb_3100_POP6_BDV1.mob
Basecaller	KB	KB	KB	KB

5 Perform an HRM Methylation Study

Perform an HRM methylation study to determine the percentage of methylated DNA in unknown samples.



Design the HRM experiment

Design and order the primers

Using Applied Biosystems Methyl Primer Express® Software, design the primers to amplify the genomic DNA that spans the methylation sites of interest. With Methyl Primer Express Software, you can specify the number of CpG dinucleotides to include in the PCR primers and their position. Order the primers from the Applied Biosystems Store.

1. Design the primers so that they meet these guidelines:

Design attribute	Design guidelines
Amplicon	• Length is less than 250 basepairs • To detect high levels of methylation, primers lie outside of the CpG island • To detect low levels of methylation, primer sequences include CpG dinucleotides
Primer length	~20 bases each
Tm	58 °C to 60 °C (Optimal Tm is 59 °C)
% GC content	30–80% GC content in each primer
3′ end	No more than 2 G+C residues in the last 5 nucleotides at the 3′ end
Repeating oligonucleotides	Avoid consecutive identical nucleotides. If you are unable to avoid consecutive identical nucleotides, make sure that each primer contains fewer than 4 consecutive Gs.

2. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user. For more instructions, see "Ordering custom primers" on page 128.

Select controls

Including controls for each target sequence in your HRM methylation study:

- At least one negative control
- Methylated DNA standards that contain from 0% to 100% methylated DNA

Optimize the HRM reactions

Optimize the HRM reactions to identify the most suitable PCR reaction to study a differentially methylated region.

1. Prepare the HRM reactions: Test different reaction conditions.
2. Amplify and melt the DNA: Review the C_T values to quantify the effficiency of the PCR reaction.
3. Review the HRM data: Review the specifity of the PCR reaction and the melting behavior of the PCR fragments.
4. Perform electrophoresis of the PCR products on high-percentage agarose gels: Verify the size of the amplicon and review the specificity of the PCR reaction.

Prepare the HRM reactions

Combine purified genomic DNA templates with MeltDoctor™ HRM Master Mix and primers to amplify the target sequence.

Note: If you are using the MeltDoctor™ HRM Reagent Kit instead of the MeltDoctor™ HRM Master Mix, see page 129 for reaction component volumes.

Prepare the methylated DNA standards

1. Obtain universally methylated DNA to represent DNA that is 100% methylated.
2. Select DNA that is non-methylated to represent DNA that is 0% methylated.
3. Mix different ratios of 100% methylated and 0% methylated DNA of equal concentration. For example:

DNA	Volume to prepare the methylated DNA standard					
100% methylated DNA (20 ng/µL)	10 µL	7.5 µL	5 µL	2.5 µL	1 µL	0 µL
Non-methylated DNA (20 ng/µL)	0 µL	2.5 µL	5 µL	7.5 µL	9 µL	10 µL
% methylated DNA	100%	75%	50%	25%	10%	0%

Note: To detect low levels of methylation, add more standards between 0% and 2% methylation. For example, prepare standards to represent 0.0%, 0.1%, 0.5%, 1%, 2%, 5%, 10%, and 100% methylation.

Treat the samples and methylated DNA standards with bisulfite

Before you perform the HRM reactions for your methylation study, treat your samples and methylated DNA standards with bisulfite to convert non-methylated cytosines (C) in your DNA to uracil (U). Samples that vary in the number of U residues within the amplified sequence will have distinct melt curve shapes and Tm values.

Applied Biosystems recommends that you use the methylSEQr™ Bisulfite Conversion Kit. For instructions, refer to the *Applied Biosystems methylSEQr ™ Bisulfite Conversion Kit Protocol* (PN 4374710).

Required materials

- Microcentrifuge tubes
- Optical reaction plate appropriate for your Real-Time PCR instrument
- MicroAmp™ Optical Adhesive Film
- MeltDoctor™ HRM Master Mix
- For each target sequence:
  - Forward and reverse primers (5 µM each)
  - DNA samples
- Methylated DNA standards
- Deionized water
- Pipettors and pipette tips
- Vortexer
- Centrifuge

Prepare the HRM reactions

Prepare the reactions for each replicate group separately, then transfer the reactions to a reaction plate appropriate for your instrument.

For information about using the MeltDoctor™ HRM Reagent Kit to optimize your reactions, see "Optimizing the reaction conditions" on page 129.

1. Prepare the negative control reactions in an appropriately sized, labeled tube:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Deionized water	7.6 µL	25.08 µL	19.0 µL	62.7 µL
Total reaction volume	**20.0 µL**	**66.00 µL**	**50.0 µL**	**165.0 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

2. Prepare methylated DNA standards and unknown reactions in separate appropriately sized, labeled tubes:

Components	384-well Fast reaction plate or 96-well Fast reaction plate		96-well standard reaction plate	
	Volume for one 20-µL reaction	Volume for three 20-µL replicates plus 10% excess	Volume for one 50-µL reaction	Volume for three 50-µL replicates plus 10% excess
MeltDoctor™ HRM Master Mix	10.0 µL	33.00 µL	25.0 µL	82.5 µL
Primer 1 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Primer 2 (5 µM)	1.2 µL	3.96 µL	3.0 µL	9.9 µL
Genomic DNA (20 ng/µL)	1.0 µL	3.30 µL	2.5 µL	8.25 µL
Deionized water	6.6 µL	21.78 µL	16.5 µL	54.45
Total reaction volume	**20 µL**	**66 µL**	**50 µL**	**165 µL**

IMPORTANT! Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

3. Vortex the reactions to mix, then spin the tubes briefly.

4. Prepare a reaction plate appropriate for your instrument:

   a. Pipet each reaction replicate to the appropriate wells of the optical reaction plate:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

   b. Seal the reaction plate with optical adhesive film, then spin the reaction plate.

   c. Confirm that the liquid is at the bottom of the wells in the reaction plate.

Note: If you plan to wait more than 24 hours before running the plate, store the plate at 4 °C. Allow the plate to warm to room temperature, then spin the plate briefly before running it.

Amplify and melt the DNA

After you have prepared the reaction plate, run the plate to amplify and melt the DNA and to generate HRM fluorescence data. This chapter contains brief instructions. For detailed instructions, see:

- Run a 384-well plate on a 7900HT Fast instrument (page 40)
- Run a 96-well plate on a 7900HT Fast instrument (page 43)
- Run a 96-well plate on a 7500 Fast instrument with 7500 Software v2.0 (page 48)
- Run a 96-well plate on a 7500 Fast instrument with SDS Software v1.4 (page 51)

Create and set up the HRM run file

Run file setting	7900HT Fast System with SDS Software v2.3 or later	7500 Fast System with 7500 Software v2.0 or later	7500 Fast Real-Time PCR System with SDS Software v1.4
Document/ experiment	• Assay: **Standard Curve (AQ)** • Container: **384 Wells Clear Plate** or **96 Wells Clear Plate** • Template: **Blank Template**	• Instrument: **7500 Fast (96 Wells)** • Experiment type: **Quantitation - Standard Curve** • Reagents: **Other**, then select the **Include Melt Curve** checkbox • Ramp speed: **Standard (~ 2 hours to complete a run)**	• Assay: **Standard Curve (Absolute Quantitation)** • Container: **96-Well Clear** • Template: **Blank Document**
Detector/target and plate layout	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**	• Reporter: **MeltDoctor** • Quencher: **None**	• Reporter: **MeltDoctor** • Quencher: **Non Fluorescent**
Plate layout	• Task for negative control wells: **NTC** • Passive Reference: **None**	• Task for negative control wells: N • Passive Reference: **None**	• Task for negative control wells: **NTC** • Passive Reference: **(none)**
Thermal profile/run method	• Mode: **Standard** • Sample Volume (µL): **20** (384-well or 96-well Fast) or **50** (96-well standard)	• Reaction Volume Per Well: **20** µL • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter-1**	• Sample Volume (µL): **20** • Run Mode: **Fast 7500** • Expert Mode: Select the checkbox • Click **Select/View Filters**, then select only **Filter A**

Run the plate

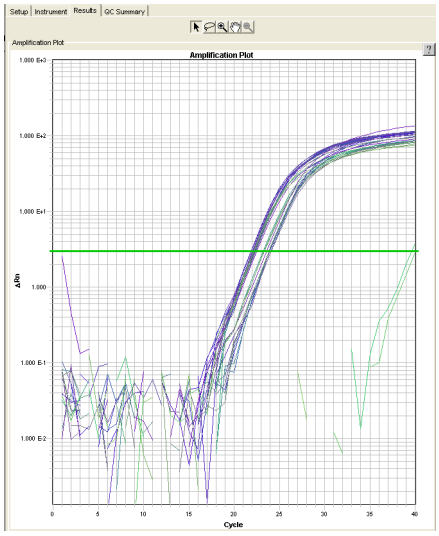
Note: If you are performing your experiment on the 7900HT Fast instrument using a 96-well reaction plate, perform the melt curve in a separate run because you need to spin the plate after you amplify the DNA.

Stage	Step	Temp	Time	Ramp rate (7900HT only)
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%

Note: For methylation experiments, adjust the annealing temperature during the amplification to increase or decrease the extent of the PCR bias.

Verify that the samples amplified and review the peaks in the melt curve

1. Review the Amplification Plot for normal characteristics:
   - Fluorescence levels that exceed the threshold between cycles 8 and 35
   - An exponential increase in fluorescence



Note: Note which wells are outlierswith C_T values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

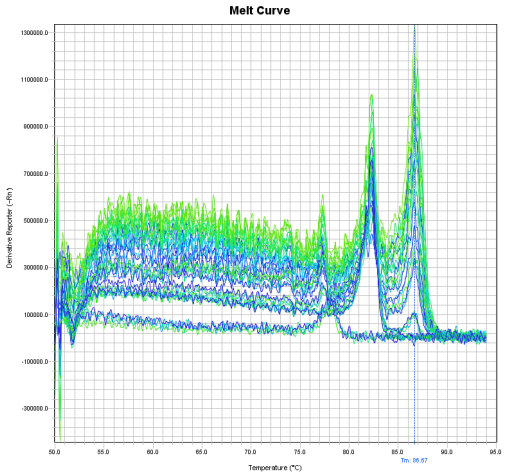
Note: If the Amplification Plot looks abnormal, refer to Chapter 6, "Troubleshooting HRM Experiments" on page 115 to identify and resolve the problem.

2. Verify that the Dissociation Curve/Melt Curve shows no unexpected Tm peaks:

   With methylation experiments, you will likely see multiple peaks. The number of peaks in the melt curve is correlated with the number of methylation sites in the amplicon.

   Note: Unexpected peaks may indicate contamination, primer dimers, or non-specific amplification.

   Note: The data appear noisy because more data is collected during a high resolution melt curve than during a standard melt curve. The extra data are required for analysis with the High Resolution Melting Software.



   The low methylation example above shows multiple Tm peaks because of the large differences between the samples with low methylation and the wild type samples. Notice that there are no large Tm peaks at the lower temperatures.

Review the high-resolution melting data

After you create, run, and analyze the *.eds or *.sds file on the 7900HT Fast or 7500 Fast system, use the Applied Biosystems High Resolution Melting Software (HRM software) to perform high resolution melting analysis of the data and review the methylation data.

Example HRM experiments

To view an example of an HRM methylation study, use the example files that are installed with the HRM software:

- High% Methylation.hrm
- Low% Methylation.hrm

The files are located in *X*:\Applied Biosystems\HRM\experiments, where *X* is the drive where you installed the HRM Software.

Create and set up the HRM experiment

For more detailed instructions on how to create and set up an HRM experiment, see pages 56 through 60.

1. Create an HRM experiment in the HRM Software using the *.eds or *.sds run file from your 7900HT Fast or 7500 Fast system.

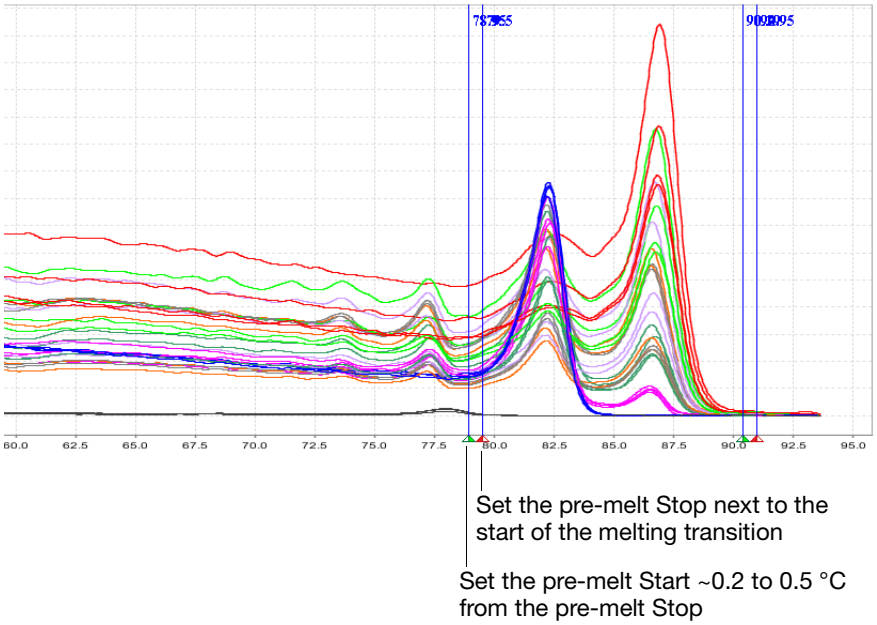
Note: If this is your first time creating an HRM experiment with the HRM software, select the default HRM calibration file. To change the HRM calibration file for a selected experiment or for all subsequent HRM experiments, see "Change the HRM calibration file" on page 131.

IMPORTANT! Use the HRM calibration file name to make sure that the HRM calibration file that is assigned to the HRM experiment is correct. The HRM calibration file must be:

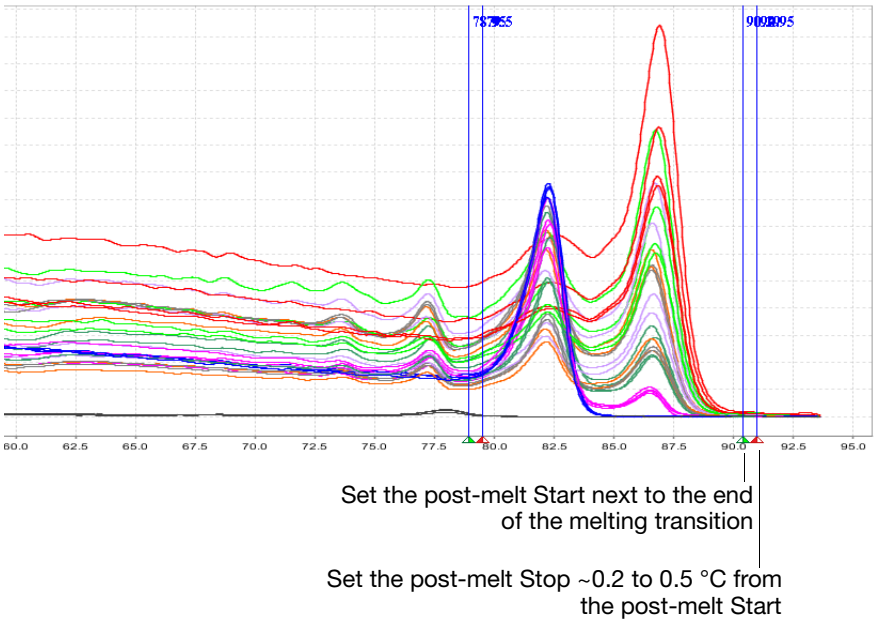
- Run on the same instrument system (the same instrument, the same block type, and the same software version) as the run file
- Run using the same HRM dye and master mix used in the HRM calibration plate

The HRM Software cannot distinguish between all calibration files. For example, it cannot distinguish between two different 7500 v2.0 instruments or between a 7900HT instrument with the Fast 96-well block and a 7900HT instrument with a standard 96-well block.

2. In the Derivative Melt Curves plot, review and adjust the pre- and post-melt regions to optimize your separation and variant calls. For most experiments, set the pre- and post-melt regions as close as possible to the melting transition region:
   - The pre-melt Start and Stop temperature lines (green and red arrows on the left) should be approximately 0.2 to 0.5 °C apart from each other.



   - The post-melt Start and Stop temperature lines (green and red arrows on the right) should be approximately 0.2 to 0.5 °C apart from each other.



3. For each control sample, enter information about that control in the HRM Software and assign the control to the appropriate wells.

   The example experiment file, Low% Methylation.hrm, contains the following controls:

Control Name	Well	Color
0%	C10	
0.1%	C9	
0.5%	C8	
1%	C7	
2%	C6	
5%	C5	
10%	C4	
100%	C3	

Note: For control names, do not use the convention *variantN*, where *N* is any number (for example, *variant1*, *variant2*, and so on). The HRM Software uses the convention *variantN* when automatically assigning the variant calls.

About the melting profiles

The melting profile of a PCR product depends on its GC content, length, sequence, and heterozygosity. High-resolution melting analysis calls variants based on the differences in the shape of the melt curves and the differences in the Tm values.

In methylation studies, the shape of the melt curve and the Tm values vary according to the number of C residues converted to U after the bisulfite treatment.

Review the populations in the Aligned Melt Curves plot

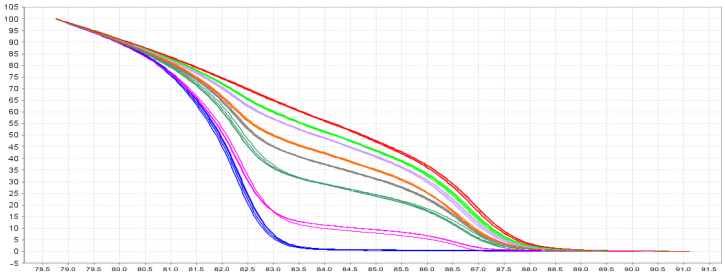
The Aligned Melt Curves plot displays the melt curves as % melt (0 - 100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set (see page 105).

1. In the Analyzed Data pane, select the **Aligned Melt Curves** tab.

2. Review:
   - Methylated DNA standards – Do the melt curves for the methylated DNA standards cluster well? Are there any outliers?
   - Define methylation range for unknowns – Which methylated standard melt curves are above and below the melt curves for the unknowns? For example, if the melt curve for an unknown sample lies between the melt curves for the 5% and 10% methylated standards, the unknown sample contains between 5% and 10% methylated nucleotides.

Aligned Melt Curves example

In the example below, there are 8 distinct variant groups, 1 for each methylation standard.



Review the Difference Plot for outliers

The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

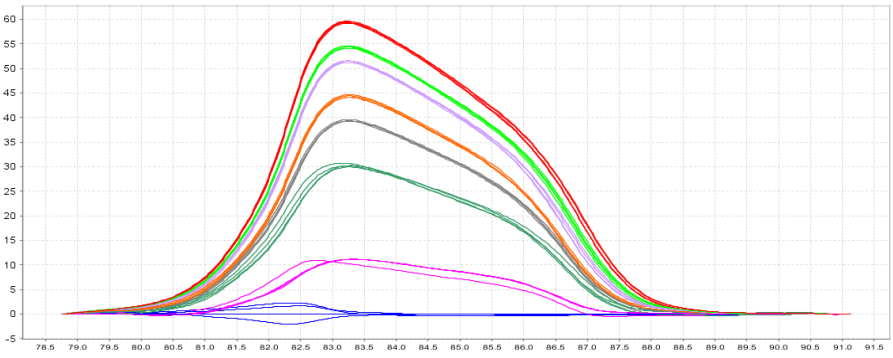
The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. In the Analyzed Data pane, select the **Aligned Data - Difference Plot** tab.
2. From the **Reference** dropdown menu, select a control or any well as the reference, then review:
   - Variant clusters – How many distinct clusters are displayed?
   - Outliers – How tight are the curves within each variant cluster?

Note: Try selecting different reference samples to find the optimal display of the clusters.

Difference Plot example

In the example below, there are 8 distinct variant groups, 1 for each methylation standard.



Review the software calls

The High Resolution Melting Software automatically makes a call for each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Results pane, click the **Well** column header to sort the results according to the well position.
2. For the methylation standard controls, review:
   - Variant Call column – Do all of the methylation standard controls have the correct call?
   - Confidence column – Are there any outliers within the replicate group? Do the values for the replicate group differ from the confidence values for the other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.
3. To view the fluorescence data for certain wells, select the rows in the Results table.

Omit outliers or change calls

After you review the software calls, you can omit outliers or change calls. Remember to click 

to reanalyze the data after you omit outliers or change calls.

For more detailed instructions, see pages 63 and 64:

- Omit outliers from analysis
- Change calls made by the software
- Revert selected manual calls to the software Auto call
- Revert all manual calls to the software Auto call

Sequence the variants

After you identify the samples that contain methylated C residues in the amplified region, dilute or purify the PCR product from the HRM reactions, then sequence the variants. Because the samples were treated with bisulfite before the HRM reactions, the U residues in the sequencing results correspond to methylated C residues.

Dilute the PCR product

1. After the PCR amplification, spin the HRM reaction plate at 100 × g for 1 minute.
2. Perform DNA quantitation of the PCR products for the selected variants, then dilute to 0.5–1.5 ng/µL with water.
3. Use the dilution ratio to determine whether you need to purify the PCR product before performing the sequencing reactions:

How much did you dilute the PCR product?	Next step
<1:20	Purify the PCR product using ExoSAP-IT® (next procedure) before performing the sequencing reactions.
>1:20	Perform the sequencing reactions using the diluted DNA (page 111).

Purify the PCR product

If you diluted the PCR product less than 1:20, purify the PCR product using ExoSAP-IT®.

1. Combine the diluted PCR product and ExoSAP-IT in a clean MicroAmp® Fast Optical Reaction Plate:

Component	Volume
Diluted PCR product	10 µL
ExoSAP-IT®	2 µL
Total reaction volume	**12 µL**

2. Mix the reactions well by pipetting up and down with a multichannel pipettor, then seal the plate with MicroAmp® Clear Adhesive Film.
3. Spin the plate at 1600 × g for 30 seconds.

4. Load the plate in the thermal cycler, cover the plate with an MicroAmp® Optical Film Compression Pad, then run the reactions in a thermal cycler:
   - Reaction volume: 12 µL
   - Thermal profile:

Stage	Temp	Time
1	37 °C	30 min
2	80 °C	15 min
3	4 °C	∞

5. After the run is complete, spin the plate at 100 × g for 1 minute.

Perform the sequencing reactions

Perform fast cycle sequencing with modifications to the protocol for the BigDye® Terminator v1.1 Cycle Sequencing Kit. If your PCR products contain an M13 tail from the primers you used in the HRM amplification reactions, use the M13F and M13R primers for the forward and reverse primers.

1. On ice, prepare 8 µL of Sequencing Master Mix for each sample:

Component	Volume
BigDye® Terminator v1.1	2 µL
Forward primer or reverse primer	1 µL
Deionized water	4 µL
BigDye® Terminator v1.1, v3.1 5X Sequencing Buffer	1 µL
Total volume per reaction	**8 µL**

Note: Include 5–10% excess volume in the master mix to compensate for pipetting error.

2. Transfer 8 µL of Sequencing Master Mix to wells of a 96-well reaction plate.

3. Add 2 µL of diluted DNA to the appropriate wells of the reaction plate, then pipet up and down to mix.

4. Seal the plate with MicroAmp® Clear Adhesive Film, then spin briefly.

5. Run the reactions in a Veriti™ 96-Well Fast Thermal Cycler:
   - Reaction volume: 10 µL
   - Thermal profile:

Stage	Step	Temp	Time
Holding	Denaturation	96 °C	1 min
Cycle sequencing (25 cycles)	Denaturation	96 °C	10 sec
	Annealing	50 °C	3 sec
	Extension	60 °C	75 sec
Holding	Holding	4 °C	∞

Note: Use a rapid thermal ramp (1 °C/second) for each new temperature.

6. After the run is complete, spin the plate briefly.

Purify the sequencing reaction

Use the BigDye XTerminator® Purification Kit to remove unincorporated BigDye® terminators. For more instructions on the purification or on transferring the plate to the DNA Analyzer, refer to the *BigDye XTerminator® Purification Kit Protocol* (PN 4374408).

1. Spin the reaction plate briefly, then add BigDye XTerminator reagents:
   a. Add 45 µL of SAM™ Solution to each reaction.
   b. Vortex the BigDye XTerminator® Solution thoroughly, then use a wide-bore pipette tip to add 10 µL to each reaction.
2. Seal the plate with MicroAmp® Clear Adhesive Film, then verify that each well is sealed.
3. Vortex the plate for 30 minutes.
4. Spin the plate at 1000 × g for 2 minutes.

Run the sequencing reaction products

Run the sequencing reaction products on an Applied Biosystems 3500/3500*xl* DNA Analyzer, 3730/3730*xl* DNA Analyzer, 3130/3130*xl* Genetic Analyzer, or 3100/3100-*Avant* Genetic Analyzer. For more instructions, refer to the user guide for your instrument.

Item	Applied Biosystems 3500/3500*xl* DNA Analyzer with 3500 Data Collection Software v1.0	Applied Biosystems 3130/3130*xl* DNA Analyzer with Data Collection Software v2.0	ABI PRISM® 3100/3100-*Avant* Genetic Analyzer with Data Collection Software v2.0
Polymer	POP-6™ polymer	POP-4™ polymer	POP-4™ polymer
Array	50 cm	36 cm	36 cm
Run file	StsSeq_BDX_50_POP6	BDX_RapidSeq36_POP4	BDX_RapidSeq36_POP4
Mobility file	Kb_3500_POP6_BDV1	Kb_3130_POP4_BDV1.mob	Kb_3100_POP4_BDV1.mob
Basecaller	KB	KB	KB

6 Troubleshooting HRM Experiments

Problems with HRM experiments are usually evidenced by abnormal amplification plots or by abnormal HRM curves.

For more guidance on troubleshooting, refer to:

- Applied Biosystems Real-Time PCR Troubleshooting Tool:
  www.appliedbiosystems.com/troubleshoot
- *Applied Biosystems Guide to High Resolution Melting (HRM) Analysis* (Stock number O-081740-0509)

Late amplification: C_T value >30 for a majority of samples

The amplification reaction may not reach the plateau phase. HRM resolution may be affected by the lower increase in fluorescence.

Possible causes	Recommended action
Poor DNA quality.	Re-extract the DNA.
Amount of DNA added to the HRM reactions is too low.	Perform PCR optimization, and increase sample input or increase the number of amplification cycles.

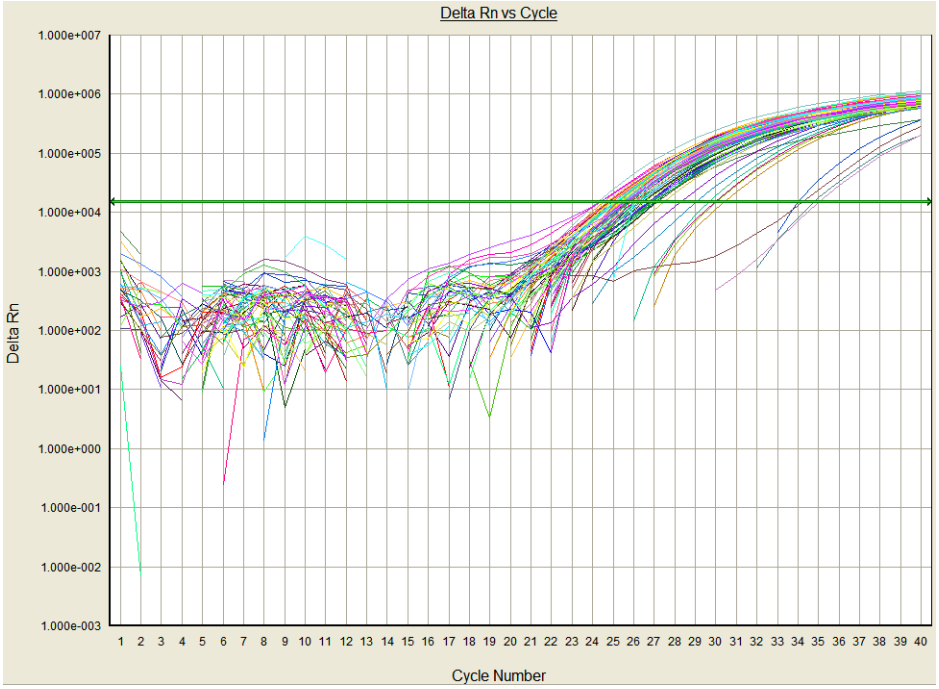
Some late amplification: C_T value >30 for some samples

Sample outliers with C_T values that are greater than those for the replicates also have a Tm shift in the HRM curve. The resulting Tm shift may affect the variant call.

Possible causes	Recommended action
Reaction volume for the outlier is visibly greater than or less than the reaction volume for the replicates.	Repeat the HRM reactions, and make sure that you add the correct volumes to each well. Also, after you seal the plate, spin the plate briefly.
Amount of DNA added to the HRM reactions is too low.	Repeat the HRM reactions with more DNA in each reaction.
PCR inhibition.	If the amplification curve also has a low slope and all replicates for a sample are affected, see page 117 to troubleshoot PCR inhibition in your HRM reactions.

PCR inhibition: Amplification curve with low slope and C_T values higher than expected

The amplification curve has a low slope and the amplification reaction may not reach the plateau phase. HRM resolution may be affected by the lower increase in fluorescence.



Possible causes	Recommended action
DNA sample contains contaminants that inhibit PCR.	Dilute the samples 1:10 or 1:100, then repeat the HRM reactions.
Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
Reaction does not contain sufficient enzyme.	Optimize the reaction using the MeltDoctor™ HRM Reagent Kit (see page 129 for reaction component volumes). You can add up to 0.15 U/µL AmpliTaq Gold® 360 DNA Polymerase to each reaction.
Reaction does not contain sufficient primer.	Optimize the reaction using the MeltDoctor™ HRM Reagent Kit (see page 129 for reaction component volumes). You can add up to 0.5 µM of each primer to each reaction.
Amplicon is greater than 200 bp.	Increase the extension timeS during the amplification reaction.
Primers are amplifying multiple targets.	Perform a BLAST search to ensure primer specificity. If the primers are not specific, design new primers.
	Reduce the number of amplification cycles.

Nonspecific amplification: Decreased PCR efficiency and multiple amplicons

Decreased PCR efficiency and multiple amplicons may affect the melting behavior of the true target amplicons.

Possible causes	Recommended action
Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
Primers are amplifying multiple targets.	Perform a BLAST search to ensure primer specificity. If the primers are not specific, design new primers.
	Reduce the number of amplification cycles.
	After PCR amplification, consider running some of the PCR product on a gel to make sure that it contains a single band.

Replicates are widely spread: Sample replicates show a wide spread in HRM curves

A wide spread within a population leads to difficulties in assessing true sequence differences, particularly between two different homozygous populations.

Possible causes	Recommended action
Population spread	Use multiple controls for HRM analysis to help you define the population spread.
Incorrect salt concentration.	Perform a $MgCl_2$ titration to find the optimal salt concentration for each reaction.
DNA starting concentrations vary widely between samples.	Make sure that the starting DNA concentrations are similar for the samples that you are testing.
Low PCR efficiencies.	Ensure efficient PCR.

Multiple melt regions: Complex melt curves with multiple melting regions

Complex melt curves are difficult to interpret. If the amplicon is too long, the melt curve may have multiple melt regions because of the regional sequence context of the amplicon.

Possible causes	Recommended action
The amplicon contains more than one SNP (genotyping experiments only).	Sequence the PCR product to confirm whether the amplicon contains more than 1 SNP. If the sequencing reveals more SNPs, redesign the primers so that the amplicon contains only 1 SNP.
The amplicon is too long.	Redesign the primers to reduce the amplicon size.

More than three different variant calls (HRM genotyping experiments only)

If the target contains unknown SNPs, multiple heterozygous and homozygous amplicons can be produced. If the amplicon is too long, the melt curve may have multiple melt regions even without a SNP because of the regional sequence context of the amplicon.

Possible causes	Recommended action
The amplicon contains more than 1 SNP.	Sequence the PCR product to confirm whether the amplicon contains more than 1 SNP. If the sequencing reveals more SNPs, redesign the primers so that the amplicon contains only 1 SNP.
The amplicon is too long.	Redesign the primers to reduce the amplicon size.

Messy HRM curves: Diagonal wavy curves below heterozygous clusters

HRM data from negative controls and unamplified samples skew the pre- and post-melt curve settings and interfere with the variant calls.

Possible cause	Recommended action
Negative controls and unamplified samples are included in the HRM analysis.	Omit negative controls and unamplified samples from the HRM analysis. Refer to the *Applied Biosystems High Resolution Melting Software Help*.

A Ordering Information

Materials and equipment for HRM calibration and HRM experiments

MeltDoctor™ HRM reagents

Item	Applied Biosystems part number
MeltDoctor™ HRM Calibration Plate, Fast 96-Well	4425618
MeltDoctor™ HRM Calibration Plate, 384-Well	4425559
MeltDoctor™ HRM Calibration Standard (20X), 1 mL	4425562
MeltDoctor™ HRM Master Mix, 5 mL bottle	4415440
MeltDoctor™ HRM Master Mix, 5 × 5 mL bottle	4415452
MeltDoctor™ HRM Master Mix, 10 × 5 mL bottle	4415450
MeltDoctor™ HRM Master Mix, 50 mL bottle	4409535
MeltDoctor™ HRM Positive Control Kit: • MeltDoctor™ HRM Allele A DNA (20X), 150 µL • MeltDoctor™ HRM Allele G DNA (20X), 150 µL • MeltDoctor™ HRM Allele A/G DNA (20X), 150 µL • MeltDoctor™ HRM Primer Mix (20X), 500 µL	4410126
MeltDoctor™ HRM Reagent Kit: • AmpliTaq Gold® 360 DNA Polymerase • AmpliTaq Gold® 360 Buffer • 360 GC Enhancer • GeneAmp® dNTP Blend • MeltDoctor™ HRM Dye (20X)	4425557

Equipment and software

Item	Source
Applied Biosystems 7500 Fast Real-Time PCR System with Notebook Computer	Applied Biosystems (PN 4351106)
Applied Biosystems 7500 Fast Real-Time PCR System with Tower Computer	Applied Biosystems (PN 4351107)
Applied Biosystems 7900HT Fast Real-Time PCR System with 384-Well Block Module	Applied Biosystems (PN 4329001)
Applied Biosystems 7900HT Fast Real-Time PCR System with 384-Well Block Module and Automation Accessory	Applied Biosystems (PN 4329002)
Applied Biosystems 7900HT Fast Real-Time PCR System with Fast 96-Well Block Module	Applied Biosystems (PN 4351405)
Applied Biosystems 7900HT Fast Real-Time PCR System with Standard 96-Well Block Module	Applied Biosystems (PN 4329003)

Item	Source
Applied Biosystems High Resolution Melting Software	Applied Biosystems (PN 4397808)
Primer Express® Software v3.0 or later	Applied Biosystems
Centrifuge with plate adapters	Major laboratory suppliers (MLS)
Lab equipment	MLS
Microcentrifuge	MLS
Microcentrifuge tubes	MLS
Pipettors and pipette tips	MLS
Vortexer	MLS

Supplies

Item	Source
Appropriate reaction plate for your instrument: • MicroAmp™ Optical 384-Well Reaction Plate with Barcode, 0.1 mL • MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL • MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL	Applied Biosystems • PN 4346906 and 4366932 • PN 4309849 and 4343814 • PN 4306737
MicroAmp® Optical Adhesive Film: • 25 covers • 100 covers	Applied Biosystems • PN 4360954 • PN 4311971
Microcentrifuge tubes	MLS
Pipettors and pipette tips	MLS

Materials and equipment for sequencing variants after HRM analysis

Equipment

Item	Source
Applied Biosystems DNA sequencer: • Applied Biosystems 3500/3500*xl* DNA Analyzer • Applied Biosystems 3730/3730*xl* DNA Analyzer • Applied Biosystems 3130/3130*xl* DNA Analyzer • Applied Biosystems 3100/3100-*Avant* DNA Analyzer	Applied Biosystems
Veriti™ 96-Well Fast Thermal Cycler	Applied Biosystems
Centrifuge with plate adapters	MLS
Microcentrifuge	MLS
Vortexer	MLS

Supplies

Item	Source
MicroAmp® Clear Adhesive Film, 100 films	Applied Biosystems PN 4306311
MicroAmp® Optical Film Compression Pad	Applied Biosystems
Wide-bore (>1 mm) pipette tips: • Wide-Orifice Tips • Clear Wide Bore Tips	 • Rainin Instrument LLC • Axygen Scientific Inc.
Microcentrifuge tubes	MLS
Pipettors and pipette tips	MLS

Reagents

Item	Source
BigDye® Terminator v1.1 Cycle Sequencing Kit, 100 reactions	Applied Biosystems (PN 4337450)
BigDye XTerminator® Purification Kit, 2 mL (~100 20-µL reactions)	Applied Biosystems (PN 4376486)
M13 forward and reverse sequencing primers: • M13 Forward (–20), 2 µg • M13 Reverse, 2 µg **Note:** Use only if the HRM PCR product contains the M13 sequences.	Invitrogen‡ • PN N520-02 • PN N530-02
UltraPure™ DNase/RNase-Free Distilled Water, 500 mL	Invitrogen PN 10977-015
ExoSAP-IT®, 100 reactions	USB Corporation‡ PN 78200
Deionized Water	MLS

‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

B Supplemental Information and Procedures

This appendix contains supplemental information and procedures for preparing and running HRM reactions and for using the High Resolution Melting Software (HRM Software).

About HRM dyes

The melting profile of a PCR product is best obtained with high-resolution melting dyes (HRM dyes). HRM dyes are double-stranded DNA(dsDNA)-binding dyes that have high fluorescence when bound to dsDNA and low fluorescence in the unbound state. HRM analysis uses dsDNA-binding dyes that are brighter than those previously used, and they do not inhibit PCR at high-dye concentrations. With traditional dyes (for example, SYBR® Green I dye), only limited concentrations of the dye can be used before the dye inhibits the PCR (Kent *et al.*, 2007).

Using other HRM dyes

This getting started guide describes procedures for calibrating your instrument and performing HRM experiments using the MeltDoctor™ HRM Dye.

If you choose to use a different HRM dye, calibrate your instrument for that dye. Follow the procedures provided, but substitute the MeltDoctor™ HRM Dye with your HRM dye and prepare your own calibration plate.

You should also optimize your reactions for the HRM dye that you choose, because each dye interacts uniquely with all other reaction components, affecting the sensitivity of the analysis.

Prepare an HRM calibration plate

This procedure is for preparing your own HRM calibration plate using the MeltDoctor™ HRM Master Mix and MeltDoctor™ HRM Calibration Standard.

IMPORTANT! The HRM calibration plate should be prepared fresh and used immediately. It is important to perform the amplification run, custom dye calibration, and HRM calibration on the same day that the HRM calibration plate is prepared.

Note: If you are using the MeltDoctor™ HRM Reagent Kit instead of the MeltDoctor™ HRM Master Mix, use the same component volumes in the HRM calibration plate that you are using in your HRM reactions.

1. Add the required volumes of each component to an appropriately sized tube:

Component	Volume (µL)					
	384-well plate		Fast 96-well plate		Standard 96-well plate	
	1 reaction	425 reactions	1 reaction	110 reactions	1 reaction	110 reactions
MeltDoctor™ HRM Master Mix	10	4250	10	1100	25.0	2750
MeltDoctor™ HRM Calibration Standard (20×)	1	425	1	110	2.5	275
Deionized water	9	3825	9	990	22.5	2475
Total volume	**20**	**8500**	**20**	**2200**	**50**	**5500**

2. Cap the tube, then vortex to mix.
3. Spin the tube briefly.
4. Pipet the HRM calibration reactions to each well of an appropriate reaction plate for your instrument:

Reaction plate	Reaction volume
Fast 384-well plate	20 µL
Fast 96-well plate	20 µL
Standard 96-well plate	50 µL

IMPORTANT! Accurate pipetting is required for proper calibration.

5. Inspect the plate to make sure all wells contain liquid.

IMPORTANT! Empty wells may cause the calibration to fail.

6. Seal the reaction plate with optical adhesive film, then spin the reaction plate.

Prepare the DNA templates

1. Purify all the DNA samples in an HRM experiment using the same method. Watch out for salt carryover because it will subtly change the thermodynamics of the DNA melting transition.

2. Perform agarose gel electrophoresis and spectrophotometry to make sure the DNA template is intact and is not contaminated with other DNAs, RNAs, proteins, or organic chemicals. Proteins and organic chemicals may inhibit the PCR amplification, and contaminating DNAs and RNAs may result in sub-optimal PCR performance or increased change of non-specific amplification.

3. Determine the quantity of DNA using spectrophotometry. If too little DNA template is added to the reaction, the fluorescence signal may not be sufficient for successful HRM analysis. If too much DNA template is added to the reaction, the PCR may be inhibited.

4. (Optional) Dilute the DNA to 20 ng/µL.

Ordering custom primers

1. Go to **www.appliedbiosystems.com**, then log into the Applied Biosystems Store if you have an account; register if you are a new user.

2. Below the Custom Primers & Probes heading, click **Custom Unlabeled Primers**, then click **Sequence Detection Primers**.

3. In the Ordering Information tab, select the check box next to the quantity of primers to order, then click **Configure**.

4. Follow the instructions on the web page to configure the primers:

   a. Select purification and formulation options.

   b. Enter or upload the primer names and sequences.

   c. Review the oligos to order.

   Note: If any of the oligos are invalid, follow the instructions on the web page to edit the sequence information.

5. Click **Add to Basket**.

6. Follow the link to your Shopping Basket, then follow the instructions on the web page to place your order.

Optimizing the reaction conditions

If you want to optimize the reaction conditions, use the MeltDoctor™ HRM Reagent Kit. See Table 1 for the recommended reaction component volumes.

For more information on optimizing your HRM reactions, refer to *A Guide to High Resolution Melting (HRM) Analysis* (Stock number O-081740 0509).

Table 1 Recommended reaction component volumes using the MeltDoctor™ HRM Reagent Kit

Components	Volume for one 20-µL reaction	Final concentration range
AmpliTaq Gold® 360 Buffer, 10×	2 µL	1×
25 mM Magnesium Chloride	1.2 to 1.8 µL	1.5 to 3.5 mM
GeneAmp® dNTP Blend, 10 mM	0.2 to 0.6 µL	100 to 300 µM each
Primer 1 (5 µM)	0.8 to 2.0 µL	0.2 to 0.5 µM
Primer 2 (5 µM)	0.8 to 2.0 µL	0.2 to 0.5 µM
MeltDoctor™ HRM Dye (20×)	1.0 µL	1×
AmpliTaq Gold® 360 DNA Polymerase (5 U/µL)	0.2 to 0.6 µL	0.05 to 0.15 U/µL
Human gDNA (20 ng/µL)	1 µL	10 pg/µL to 10 ng/µL
Deionized water	As needed	–
Total volume	**20 µL**	

Computer requirements

The hardware and software requirements for running version 2.0 of the HRM software are listed in the table below.

Component	Recommended Requirements	Minimum Requirements‡
Computer	• Intel® Pentium® 4 processor or compatible processor, 2.0 GHz • 1 GB of RAM • One hard drive§ with 10 GB available • 20/48X IDE CD-ROM • USB v2.0 • Ethernet network interface adapter (10BASE-T) • UL listed • CE marked • FCC labeled	• Intel® Pentium® 4 processor or compatible processor, 1.2 GHz • 1 GB of RAM • One hard drive with 10 GB available • 20/48X IDE CD-ROM • USB v1.1 • Ethernet network interface adapter (10BASE-T) • UL listed • CE marked • FCC labeled
Monitor	• 1280 × 1024 pixel resolution for full screen display • 16-inch color monitor • 32-bit color • UL listed	• 1280 × 1024 pixel resolution for full screen display • 16-inch color monitor • 32-bit color • UL listed
Operating System	• Microsoft® Windows® XP Operating System, Service Pack 1 or greater • Microsoft® Windows® Vista® Operating System	Microsoft® Windows® XP Operating System, Service Pack 1 or greater

‡ The Minimum Requirements column lists the lowest specifications that permit the installer to install the software. The minimum requirements may not provide optimal performance. Applied Biosystems does not guarantee support of an installation in this environment.

§ For optimal performance of the software, partition the hard drives on your computer.

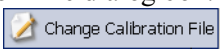
Change the HRM calibration file

Use the Change Calibration File function to:

- Change (overwrite) the HRM calibration file for a selected HRM experiment
- Change the default HRM calibration file for all subsequent new HRM experiments

Change (overwrite) the HRM calibration file

This option changes (overwrites) the HRM calibration file for the *selected* HRM experiment.

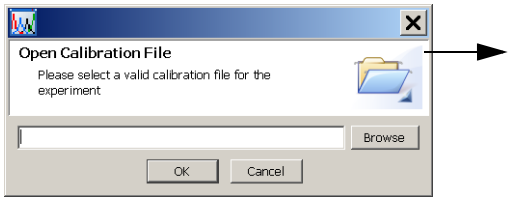
1. In the HRM Experiments pane, select the HRM experiment to edit.
2. Open the Open Calibration File dialog box:
   - In the toolbar, click 

     or
   - In the menu bar, select **File ▸ Change Calibration File**.
3. Browse to and select the appropriate HRM calibration file (*.eds or *.sds file), then click **OK**.

IMPORTANT! The HRM calibration file must be:

- Run on the same instrument as the run file
- Run in the same reaction plate type as the run file (384-well, 96-well Fast, or 96-well standard)
- Run with the same software version as the run file

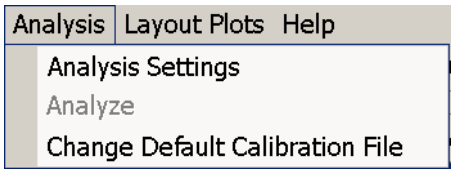
Note: If you cannot see the Browse button, resize the dialog box.



Change the default HRM calibration file

This option changes the default HRM calibration file for *all subsequent new* HRM experiments.

1. From the menu bar, select **Analysis ▸ Change Default Calibration File** to open the Open Calibration File dialog box.



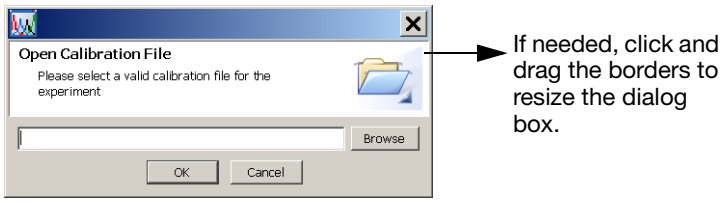
2. From the Instrument Type dropdown menu, select the appropriate instrument.

3. Browse to and select the appropriate HRM calibration file (*.eds or *.sds file), then click **OK**.

IMPORTANT! The HRM calibration file must be:

- Run on the same instrument as the run file
- Run in the same reaction plate type as the run file (384-well, 96-well Fast, or 96-well standard)
- Run with the same software version as the run file

Note: If you cannot see the Browse button, resize the dialog box.



If you are running multiple HRM assays

In the HRM Software, an assay is defined as a specific combination of Detector/Target and Dye. You can have multiple assays in the same plate, and the HRM Software analyzes each assay separately, using distinct analysis settings.

If your reaction plate contains multiple HRM assays, make sure that you select the assay from the Assay dropdown menu before you review the results or make any changes to the HRM experiment file.

Publishing the data

You can publish the data from the HRM Software in several ways:

- Copy and paste data from the Results Pane to a text or spreadsheet application (for example, Microsoft® Word Software or Microsoft® Excel Software).
- Create slides
- Export data
- Print a plot
- Print a report
- Save a plot as an image file

For information on performing these procedures, click  **or press F1** in the software to access the software Help.

C Software Warranty Information

Computer Configuration

Applied Biosystems supplies or recommends certain configurations of computer hardware, software, and peripherals for use with its instrumentation.
Applied Biosystems reserves the right to decline support for or impose extra charges for supporting nonstandard computer configurations or components that have not been supplied or recommended by Applied Biosystems. Applied Biosystems also reserves the right to require that computer hardware and software be restored to the standard configuration prior to providing service or technical support. For systems that have built-in computers or processing units, installing unauthorized hardware or software may void the Warranty or Service Plan.

Limited Product Warranty

Limited Warranty

Applied Biosystems warrants that for a period of ninety (90) days from the date the warranty period begins, its High Resolution Melting Software will perform substantially in accordance with the functions and features described in its accompanying documentation when properly installed on the instrument system for which it is designated, and that for a period of ninety (90) days from the date the warranty period begins, the tapes, diskettes, or other media bearing the software product will be free of defects in materials and workmanship under normal use. If buyer believes that it has discovered a failure of the software to satisfy the foregoing warranty, and if buyer notifies Applied Biosystems of such failure in writing during the ninety (90) day warranty period, and if Applied Biosystems is able to reliably reproduce such failure, then Applied Biosystems, at its sole option, will either (i) provide any software corrections or “bug-fixes” of the identified failure, if and when they become commercially available, to buyer free of charge, or (ii) notify buyer that Applied Biosystems will accept a return of the software from the buyer and, upon such return and removal of the software from buyer's systems, terminate the license to use the software and refund the buyer's purchase price for the software. If there is a defect in the media covered by the above warranty and the media is returned to Applied Biosystems within the ninety (90) day warranty period, Applied Biosystems will replace the defective media. Applied Biosystems does not warrant that the software will meet buyer's requirements or conform exactly to its documentation, or that operation of the software will be uninterrupted or error free.

Warranty Period Effective Date

Any applicable warranty period under these sections begins on the earlier of the date of installation or ninety (90) days from the date of shipment for software installed by Applied Biosystems personnel. For all software installed by the buyer or anyone other than Applied Biosystems, the applicable warranty period begins the date the software is delivered to the buyer.

Warranty Claims

Warranty claims must be made within the applicable warranty period.

Warranty Exceptions

The above warranties do not apply to defects resulting from misuse, neglect, or accident, including without limitation: operation outside of the environmental or use specifications, or not in conformance with the instructions for the instrument system, software, or accessories; improper or inadequate maintenance by the user; installation of software or interfacing, or use in combination with software or products, not supplied or authorized by Applied Biosystems; and modification or repair of the product not authorized by Applied Biosystems.

The foregoing provisions set forth Applied Biosystems' sole and exclusive representations, warranties, and obligations with respect to its products, and Applied Biosystems makes no other warranty of any kind whatsoever, expressed or implied, including without limitation, warranties of merchantability and fitness for a particular purpose, whether arising from a statute or otherwise in law or from a course of dealing or usage of trade, all of which are expressly disclaimed.

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The remedies provided herein are the buyer's sole and exclusive remedies. Without limiting the generality of the foregoing, in no event shall Applied Biosystems be liable, whether in contract, tort, warranty, or under any statute (including without limitation any trade practice, unfair competition, or other statute of similar import) or on any other basis, for direct, indirect, punitive, incidental, multiple, consequential, or special damages sustained by the buyer or any other person or entity, whether or not foreseeable and whether or not Applied Biosystems is advised of the possibility of such damages, including without limitation, damages arising from or related to loss of use, loss of data, failure or interruption in the operation of any equipment or software, delay in repair or replacement, or for loss of revenue or profits, loss of good will, loss of business, or other financial loss or personal injury or property damage.

No agent, employee, or representative of Applied Biosystems has any authority to modify the terms of this Limited Warranty Statement or to bind Applied Biosystems to any affirmation, representation, or warranty concerning the product that is not contained in this Limited Warranty Statement, and any such modification, affirmation, representation, or warranty made by any agent, employee, or representative of Applied Biosystems will not be binding on Applied Biosystems unless in a writing signed by an executive officer of Applied Biosystems.

This warranty is limited to the buyer of the product from Applied Biosystems and is not transferable.

Some countries or jurisdictions limit the scope of or preclude limitations or exclusion of warranties, of liability, such as liability for gross negligence or willful misconduct, or of remedies or damages, as or to the extent set forth above. In such countries and jurisdictions, the limitation or exclusion of warranties, liability, remedies or damages set forth above shall apply to the fullest extent permitted by law, and shall not apply to the extent prohibited by law.

D Safety

This appendix covers:

General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page 139.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

MSDSs

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Biological hazard safety

General biohazard

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

www.cdc.gov

Bibliography

Giffard, P. M., Huygens F., Price, E. P., and Smith, H. May 2007. High-Resolution DNA Melt Curve Analysis of the Clustered, Regularly Interspaced Short-Palindromic-Repeat Locus of *Campylobacter jejuni*. *Applied and Environmental Microbiology*, 73: 3431–3436.

Graham, R., Liew, M., Lyon, E., Meadows, C., and Wittwer, C.T. 2005. Distinguishing Different DNA Heterozygotes by High-Resolution Melting. *Clinical Chemistry*, Vol. 51, No. 7, 1295–1298.

Kent, J.O., Reed G. H., and Wittwer, C.T. June 2007. Review: High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*, Vol. 8, No. 6, 597–608.

Related documentation

HRM documentation

The following documents about HRM experiments are available from Applied Biosystems:

- *Applied Biosystems High Resolution Melting Software Help* (PN 4393101) – Installed with and accessible from within the HRM Software, the Help system describes the software and provides procedures for common tasks.
- Quick reference cards provide abbreviated procedures for performing HRM experiments using MeltDoctor™ HRM Reagents and HRM Software:
  - *Perform an HRM Genotyping Experiment Quick Reference Card* (PN 4421675)
  - *Perform an HRM Mutation Scanning Experiment Quick Reference Card* (PN 4426174)
  - *Perform an HRM Methylation Study Quick Reference Card* (PN 4426173)
- *A Guide to High Resolution Melting (HRM) Analysis* (Stock number O-081740) – This document provides background information on HRM analysis to help you perform robust HRM experiments.

Access the HRM Help system

To access the *Applied Biosystems High Resolution Melting Software Help* system from within the HRM software, click  in the software window, select **Help ▸ HRM Help**, or press **F1**.

7500 Fast and 7900HT Fast System documentation

The following related documents are available from Applied Biosystems:

Instrument and software	Document	Part number
7900HT Fast System and SDS Software v2.3 or later	*Applied Biosystems 7900HT Fast Real-Time PCR System Maintenance and Troubleshooting Guide*	4365542
	Sequence Detection Systems Software Online Help for the Applied Biosystems 7900HT Fast Real-Time PCR System	NA‡
7500 Fast System and 7500 Software v2.0 or later	*Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide*	4387777
	Applied Biosystems 7500 Software v2.0 Help	NA
7500 Fast System and SDS Software v1.4	*Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide*	4347828
	Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Online Help	NA

‡ The instrument Help systems can be accessed from within the instrument software.

How to obtain support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

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